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[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 1~4, 1957]

Studies on the Metabolic Products of *Macrosporium porri* Elliott

Part I. Isolation and General Properties of Macrsporin, A New Pigment

By Rikisaku SUEMITSU, Yukio MATSUI, and Makoto HIURA

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Received February 11, 1956

A strain of *Macrosporium porri* Elliott, when grown at 25° on 2% glucose solution of stone-leek decoction, gives rise to a hitherto undescribed pigment to which the name "macrosporin" is proposed. Optimum cultural condition for the production of the pigment and the method of its isolation are described here. Macrosporin, $C_{16}H_{12}O_5$, crystallizes as orange-yellow rhombic and melts at 300–302° with decomposition. General properties of macrosporin, its diacetate, m.p. 209–210°, and trimethylether, m.p. 260–261°, are described. Macrosporin is recognized to be a hydroxyanthraquinone derivative from the results of elementary analysis, molecular weight determination, and ultra-violet and infra-red spectra.

In the previous literature, there are many reports concerning the pigments produced by species of *Penicillium*, *Helminthosporium*, *Aspergillus*, and *Fusarium*. However, nothing has been reported, either on the pigments, or on the metabolic products of *Macrosporium*. Two crystalline pigments were isolated from the mycelia of *Macrosporium porri* Elliott, which is known as a pathogenic organism causing the black spot disease of stone-leek, cultured in liquid media for 25 days. For one of them, hitherto undescrived, the name "macrosporin" is proposed in connection of the species name, while the other pigment will be described later. Macrosporin, $C_{16}H_{12}O_5$, m.p. 300–302° (decom.) is an orange-yellow pigment having anthraquinone-like properties. It is easily soluble in N-NaOH and N-Na_2CO_3 to give purple-red solution and, on acidifying with HCl the pigment is regenerated.

It dissolves in conc. H_2SO_4 to give an orange-red solution. It dissolves very sparingly in almost all of the organic solvents. Its glacial acetic acid solution is yellow in color, and does not show fluorescence in daylight. The color disappears on reducing with zinc dust and the color is restored by standing for a while. This color-reaction is characteristic of the *p*-quinone type compounds. Its alcoholic solution gives dark-brown color with ethanolic $FeCl_3$ and orange-yellow color with ethanolic magnesium acetate[1].

The ultra-violet absorption spectrum shows absorption bands, λmax. 225 mμ (log. 4.32) indicating dihydroxyanthraquinone[2] structure, and λmax. 285 mμ (log. 4.30)[3] also indicating the characteristic of quinoid nucleus of anthraquinone besides a broad band λmax. 381 mμ (log. 3.90).

The infra-red spectrum of macrosporin gives a non-chelated carbonyl band at 6.02 μ, chelated carbonyl band at 6.11 μ, and a band by the hydroxyl group located the β-position to carbonyl group at 3.04μ[4]. As it is apparent that in every case, when a hydroxyl group not adjacent to a carbonyl group is present, a band is to be obserbed at about 2.99 μ, and macrosporin is believed to contain a hydroxyl

1) S. Shibata, et al., *J. Am. Chem. Soc.*, **72**, 2789 (1950).
2) T. Ikeda, et al., *J. Pharm. Soc. Japan*, **76**, 217 (1956).
3) L.H. Briggs, et al., *J. Chem. Soc.*, **1952**, 1718.
4) M. St, C. Flett *J. Chem. Soc.*, **1948**, 1441.

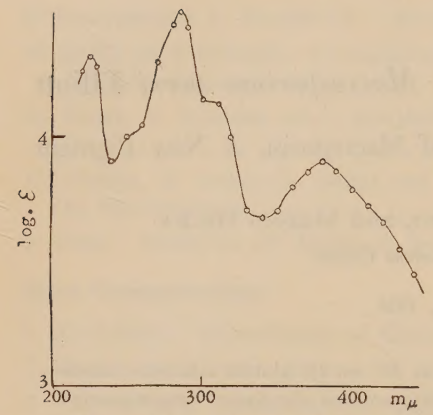

FIG. 1. UV-absorption Spectrum of Macrosporin.
(5×10^{-5} mol ethanol solution)

Judging from its general properties and both ultra-violet and infra-red absorption data of macrosporin, it is very probable that macrosporin may be a hydroxyanthraquinone derivative represented by formula, $C_{16}H_{12}O_5$, involving 1 methoxy group and 2 hydroxyl groups, one of which may be located at least in the β-position to the carbonyl group.

Six of the pigments produced by micro-organisms have been previously reported to involve the methoxy group. They are: roseopurpurin (4-methoxy-emodin), phycion (7-methoxy-emodin), erythroglaucin (7-methoxy-catenarin), nalgiovensin (4,5-dihydroxy-7-methoxy-2-hydroxypropylanthraquinone), nalgiolaxin (1 or 8-chloronalgiovensin), and carviolin (4-methoxy-ω-hydroxyemodin). Though a few hydroxyanthraqninone[6,7,8] derivatives having the methoxy group have been obtained from other sources they are not identical with macrosporin.

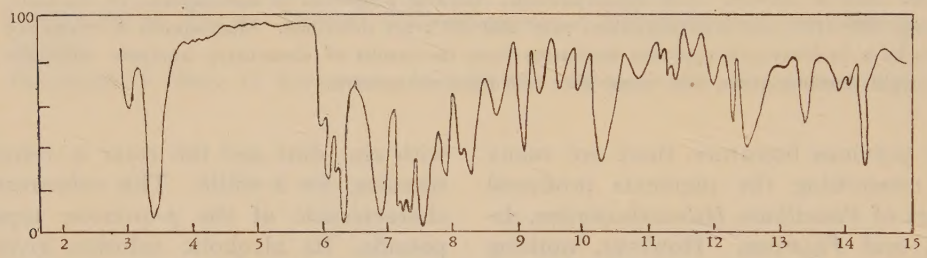

FIG. 2. Infra-red Absorption Spectrum of Macrosporin.

group β-positioned to carbonyl group in it.

On treatment with acetic anhydride in the presence of a trace amount of conc. H_2SO_4, macrosporin gives diacetate, $C_{16}H_{10}O_5(COCH_3)_2$, light yellow needles, m.p. 209–210° and on boiling with dimethyl sulfate and K_2CO_3 in acetone, it gives a trimethylether $C_{15}H_7O_2(OCH_3)_3$, yellow needles, m.p. 260–261°. On determination of the methoxy group by Zeisel's method both macrosporin and diacetate give 1 methoxy group respectively, while trimethylether gives 3 methoxy groups.

Some reports concerning the paper chromatography of hydroxyanthraquinone[1,5] have been given by Shibata et al. By using Shibata's solvent system it was found that an orange spot of R_F 0.37 is given only with butanol saturated with 28% NH_4OH.

EXPERIMENTAL

Culture. The strain of *Macrosporium porri* Elliott used in this work, was obtained from the experimental field attached to our college.

Cultural Condition. Two % glucose solution of stone-leek decoction was used as the cultural medium. A number of 500-ml Erlenmyer flasks, containing 150 ml of the solution, pH 6.5–7.0, were sterillized and inoculated with the strain which had been freshly cultured. The inoculated flasks were incubated at 25° in the dark. On the 5th day the mycelia were light-cream in color and covered the surface of the

5) M. Takido, *Pharm. Bull.*, **4**, 45 (1956).

6) R. Hill, *Nature*, **134**, 628 (1934).

7) A.G. Perkin, *J. Chem. Soc.*, **91**, 2066 (1907).

8) S. Nonomura, *J. Pharm. Soc. Japan*, **75**, 219 (1955).

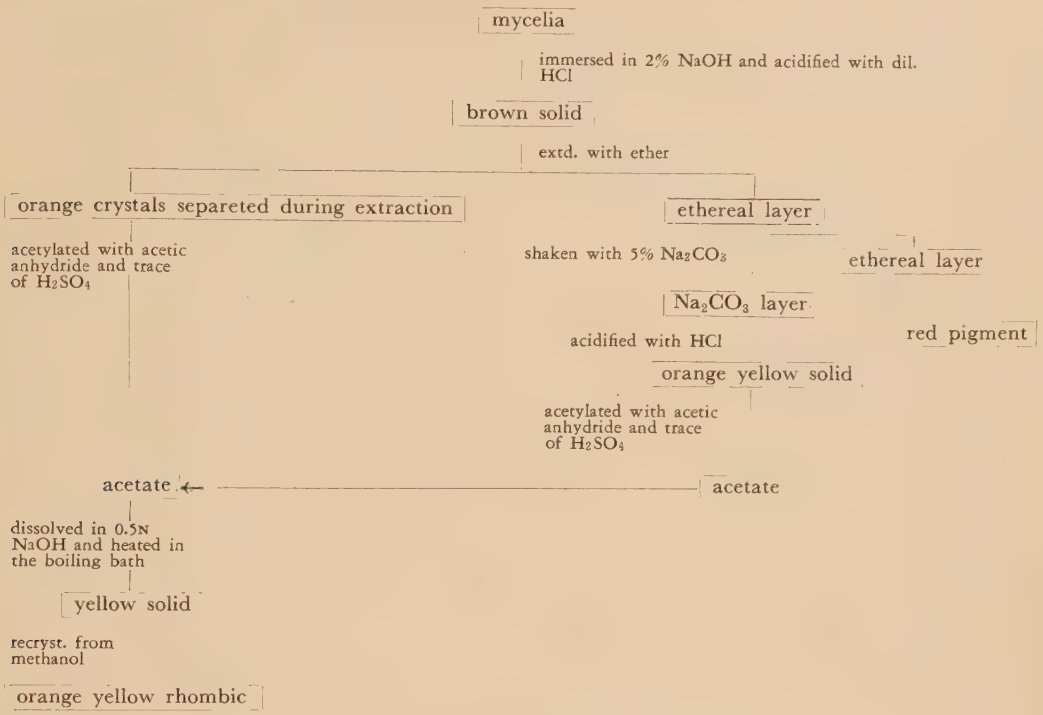

FIG. 3. Isolation and Purification of the Pigment.

medium, and became yellow-orange on the 10th day. When harvested, at the end of 25 days, the mycelia became dark-red in color and the solution also turned dark-red.

Isolation and Purification of the Pigment. The mycelia were filtered by cloth, dried at 45° and powdered. The method of purification of the pigment is given in Fig. 3.

As it was doubtful that the pigment obtained by the method described in Fig. 3 might undergo change by NaOH treatment, the dried mycelia were directly extracted with ether and in this way the yellow-orange pigment was obtained. The pigment was identical with the one which was mentioned before. About 0.6 g of crystalline pigment was obtained from 10.4 l of substrate and its weight was about 2.5% of the dried mycelium.

General Properties of Macrosporin. Macrosporin crystallizes from glacial acetic acid as beautiful orange-yellow rhombic, m.p. 300–302° (decomp.) *Anal.* Found: C, 67.40; H, 4.39; OCH_3, 10.17; Cald. for $C_{15}H_9O_4$-OCH_3: C, 67.60; H, 4.26; OCH_3, 10.91.

It readily sublimes at about 280°, does not dissolve

FIG. 4. Crystals of Macrosporin. (× 640)

in sat. $NaHCO_3$, but dissolves in N-Na_2CO_3 to a purple-red solution and readily dissolves in N-NaOH to a deep purple-red solution. Its ethanolic solution gives a darkbrown color with ethanolic $FeCl_3$ and orange-yellow color with ethanolic magnesium acetate. It dissolves in conc. H_2SO_4 to an orange-red solution. Its glacial acetic acid solution is yellow in color, and does not give rise to fluoresence in daylight. Macrosporin hardly dissolves in ether, benzene, chloroform and toluene, but dissolves in hot acetone, hot ethanol and hot glacial acetic acid.

Macrosporin Diacetate. Macrosporin (0.1 g) was dissolved in acetic anhydride (10 ml) and a drop of conc. H_2SO_4 was added. It was then cooled and poured into water. The dark-yellow solid separated was filtered, dried and crystallized from methanol to give fine light needles (0.07 g), m.p. 209–210°. *Anal.* Found: C, 65.26; H, 4.52; OCH_3, 8.23; M.W. (Rast) 371; Cald. for $C_{15}H_7O_5(COCH_3)_2OCH_3$: C, 65.21; H, 4.38; OCH_3 8.42; M.W. 368.

Macrosporin Trimethylether. A mixture of macrosporin (0.55 g), anhydrous K_2CO_3 (30 g), redistilled dimethyl sulfate (4 ml) and dry acetone (200 ml) was boiled under reflux. After boiling for 3 hrs., anhydrous K_2CO_3 (15 g) and dimethyl sulfate (4 ml) were added and the reaction was continued further for a period of 7 hours. The hot solution was filtered and evaporated to a yellow solid, which was recrystallized from acetone and finally from methanol to give yellow needles (0.4 g), m.p. 260–261°. *Anal.* Found: C, 69.09; H, 5.17; OCH_3, 28.30; Cald. for $C_{15}H_7O_2(OCH_3)_3$: C, 69.22; H, 5.16; OCH_3, 29.81.

Paper Chromatography of Macrosporin. Ethanol solution of the pigment was placed on a filter paper (Toyo Roshi No. 3, 1.5×25) and developed at 18° by one-dimentional decending method using butanol saturated with 28% NH_4OH as the developing solvent. The developed paper was dried and the spot was examined. color; orange, R_F; 0.37.

We are indebted to Prof. Igarashi of Hokkaido Univ., Prof. Takei of Kyotó Univ., for their good suggestions and Prof. Mitsui of Kyoto Univ. for the elementary analysis.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 5~10. 1957]

Studies on Synthetic Pyrethroids

Part IX. Assignment of the Geometrical Configuration to $\alpha\delta$-Dimethylsorbic Acid

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Received July 25, 1956

The geometrical configuration of $\alpha\delta$-dimethylsorbic acid, an important intermediate in our successful total synthesis of chrysanthemum-dicarboxylic acid, is confirmed to be *trans* by the ultra-violet and infra-red absorptions and also by the dissociation constant of $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid derived from the parent $\alpha\delta$-dimethylsorbic acid by semi-hydrogenation over a palladium catalyst. Thus, the *trans*-configuration of this acid, previously deduced on a theoretical basis, is free from any experimental defect, and thence, the *trans*-configuration of the side-chain double-bond of the naturally derived chrysanthemum-dicarboxylic acid follows from the method of synthesis from *trans*-$\alpha\delta$-dimethylsorbic acid. This method of assignment may, in general, be applicable to the α-alkyl substituted conjugated diene carboxylic acids.

In the course of our total synthesis of chrysanthemum-dicarboxylic acid[1], methyl or ethyl $\alpha\delta$-dimethylsorbate which was at first obtained from the dehydration of the parent Reformatski ester, was employed as an olefinic component for the addition of ethyl diazoacetate. The geometrical configuration of the $\alpha\beta$-double-bond in this molecule was regarded as *trans*, by stereochemical consideration on the atomic model of this acid, but experimental evidence for the *trans*-configuration to this acid has been lacking as yet.

The experimental assignment of this configuration is of importance, because this configuration is retained as such[1a] during the addition of ethyl diazoacetate to produce the synthetic chrysanthemum-dicarboxylic acids, one of which (m.p. 208°) has been identified as the racemic form of the naturally derived chrysanthemum-dicarboxylic acid and the dextrorotatory acid obtained by the optical resolution has been identified with the natural acid[1c], thereby establishing hitherto unknown configuration of the side-chain double-bond of the naturally derived chrysanthemum-dicarboxylic acid to be *trans*.

Linstead and his co-workers[2] have recently summarized evidence to establish the geometrical configurations of the isomeric sorbic acids, showing that partial inversion to *cis-trans* markedly affects the ultra-violet light absorption. The ultra-violet light absorption of the only known form of $\alpha\delta$-dimethylsorbic acid shows the single high intensity band (λmax. 273 mμ, ε 22,300) that would surmise the *trans*-structure. However, even if the bathochromic shift produced by two additional methyl substituents in α- and δ-positions of sorbic acid is allowed, it seems subject to uncertainty to decide the configuration of $\alpha\beta$-double-bond to be *trans* by applying Linstead's spectral data to our acid, because of the absence of geometrical isomerism with reference to the $\gamma\delta$-double-

1) Y. Inouye, Y. Takeshita and M. Ohno, (a) This Bulletin, **19**, 193 (1955), (b) *Bull. Inst. Chem. Res. Kyoto Univ.*, **33**, 73 (1955), (c) *ibid*, **34**, 90 (1956), (d) *Botyu Kagaku*, **20**, 102 (1955)

2) U. Eisner, J.A. Elvidge, R.P. Linstead, *J. Chem. Soc.*, **1953**, 1372

bond in contrast to the isomeric sorbic acids in which geometrical isomerism exists in both double-bonds.

So far, no reliable method for assigning geometrical configuration to the alkyl substituted sorbic acids has been developed.

In the present paper, are described the partial hydrogenation of $\alpha\delta$-dimethylsorbic acid to give $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid and the assignment of geometrical configuration thereof by the infra-red and ultra-violet light absorptions and by measurement of the dissociation constant.

Farmer and Hughes[3] reported that sorbic acid, when submitted to 50% hydrogenation in the presence of a palladium catalyst, yielded a mixture of dihydro-acids in which the $\Delta\alpha$-dihydrosorbic acid predominated ranging from 85–90%, whilst with a platinum catalyst, only a mixture of an almost equal amount of fully reduced and unchanged conjugated compounds was obtained, the production of smaller proportions (below 20%) of dihydro-compounds being recorded.

The semi-hydrogenation of $\alpha\delta$-dimethylsorbic acid over a Pd–$BaSO_4$ catalyst predominantly afforded the $\Delta\alpha$-dihydrocompound as was expected, and repeated purification by rectifications and partial esterifications* gave the pure $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid, which was characterized by ozonolysis to yield pyruvic acid (as a 2,4-dinitrophenylhydrazone, m.p. 218°) and *iso*-valeric acid (as itself and as a *p*-phenylphenacylester, m.p. 77°). In the ultra-violet light absorption spectrum of the resulting $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid, the occurrence of the single intense band (λmax. 218 mμ, ε 14, 300), characteristic for the $\alpha\beta$-unsaturated carboxylic chromophore, together with the disappearance of the conjugated diene carboxylic band (λmax. 273mμ, ε 22,300) which was exhibited by the parent dimethylsorbic acid, were in good agreement with the chemical evidence mentioned above. The fact that the extinction coefficient at this band did not alter on further purifications, excluded the contamination of the possible $\Delta\beta\gamma$-, $\Delta\gamma\delta$-dihydro or fully reduced compounds which are spectrally inert in this region. This acid was quantitatively hydrogenated over a platinum catalyst and was shown to absorb one equivalent hydrogen, yielding the known $\alpha\delta$-dimethylcaproic acid[1d]. The equivalent weight and the acid dissociation constant were also determined. All criteria supported purity of this acid.

The stereomutation during the semi-hydrogenation over a palladium catalyst has not been observed so far as is known, and the configuration of the $\alpha\beta$-double-bond in the parent conjugated diene acid is believed to be retained during the semi-hydrogenation. For example, ordinary *trans-trans*-sorbic acid, when it undergoes the same reduction sequence, affords solid $\Delta\alpha$-dihydrosorbic acid[3] m.p. 34°, which is completely identical with the *trans* acid obtained from the Doebner condensation of malonic acid with butyraldehyde in pyridine[4,5], in contrast to another liquid isomeric acid[6] (b.p. 201°), obtained from the semi-hydrogenation of pentin-1-carboxylic acid over colloidal palladium, that the *cis*-configuration is favoured in general from this reduction sequence.

Furthermore, the stereomutation during any of the procedures employed throughout the purification could be excluded, for this acid was not submitted to the thermal conditions likely to invert the configuration. Reference has been made to the thermodynamic stability of angelic acid[7], and to the thermal equilibration data[8] on isomeric

3) E.H, Farmer, L.A. Hughes, *J. Chem. Soc.*, **1934**, 1929.

* In the sense that $\Delta\beta$- and $\Delta\gamma$-dimethylhexenoic, dimethylcaproic acids become esterified, whilst $\Delta\alpha$-dimethylhexenoic acid remains unchanged. (Compare Ecott and Linstead's modification of Sudborough's method, *J. Chem. Soc.*, **1929**, 2153; **1932**, 125).

4) Walbaum, Rosenthal, *J. pr.*, [2], **124**, 65

5) A.A. Goldberg, R.P. Linstead, *J. Chem. Soc.*, **1928**, 2351

6) Bourguel, Yvon, *C.r.*, **182**, 224; **188**, 1494, *Bl.*, [4], **45**, 1077

7) W.G. Young, R.T. Dillon, H.J. Lucas, *J. Am. Chem. Soc.*, **51**, 2528 (1929)

8) J. Cason, M.J. Kalm, *J. Org. Chem.*, **19**, 1947 (1954)

2-methyl-2-hexenoic acids. At this molecular weight, approximately the same order of magnitude as that of our acid, the isomeric acids proved to be sufficiently stable to heat (temperatures involved being in the range as high as 220–270°) to allow purification by fractional distillation under a reduced pressure.

Naturally, it is desirable to examine both geometrical isomers in comparison, if possible, before making the assignment, but the only one known form is available in the present case.

Fortunately, spectral data[8] of some homologous geometrical isomers of well-established configuration are available for reference. In Fig. 1 are shown the ultra-violet absorption spectra of $\alpha\delta$-dimethyl-$\Delta\alpha$-

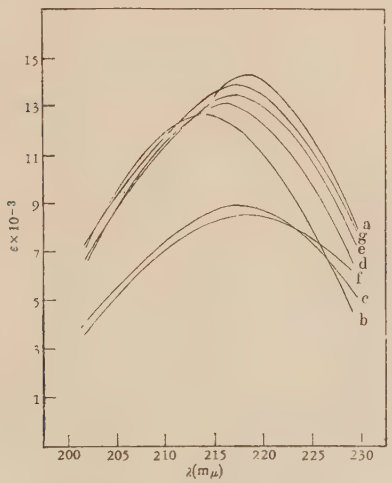

FIG. 1. Ultra-violet Light Adsorption Spectra of 2-Methyl-2-alkenoic Acids.

(a) $\alpha\delta$-Dimethyl-$\Delta\alpha$-hexenoic acid, (b) Tiglic acid, (c) Angelic acid (d) *trans*-2-Methyl-2-pentenoic acid, (e) *trans*-2-Methyl-2-hexenoic acid, (f) *cis*-2-Methyl-2-hexenoic acid, (g) *trans*-2-Methyl-2-heptenoic acid. (b-g) J. Cason and M. J. Kalm, *J. Org. Chem.*, **19**, 1947 (1954).

hexenoic acid, together with those of the homologous C_5–C_7 α-methyl-$\alpha\beta$-unsaturated acids given by Cason[8]. It was somewhat surprising that the ultra-violet light absorption maxima of both isomers were at essentially the same wave-length (217–218 mμ), unlikely to serve as a decisive criterion, but the extinction coefficients showed a significant difference, those of *cis*-isomers being much lower in every case. $\alpha\delta$-Dimethyl-$\Delta\alpha$-hexenoic acid has an extinction coefficient of 14,300 and λmax. at 218 mμ. Since steric interference between carboxyl and terminal alkyl may be regarded as the cause of the less intense absorption of the *cis* isomer, the small decrease in absorption accompanying change from terminal methyl to a larger group may be interpreted as indicating only small steric interference[9] of the substituent beyond the *gamma* position. Thus, steric influence of the branched-chain substituent beyond the *gamma* position of $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid could be spectrally negligible in the case of comparison with the straight-chain homologues (Fig. 1. b-g), and it is, therefore, concluded from the spectral properties given above that this acid is of the *trans* acid series.

In Fig. 2, are assembled the infra-red spectra of $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid together with those of several homologous isomeric acids prepared by Cason[8].

At a glance, the differences in these curves are not so characteristic as to distinguish the two isomeric acids, but elaborate inspection of the fine structures indicates the regions of clean-cut distinction. For simplicity, the wave lengths of the bands of $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid in comparison with those of isomeric 2-methyl-2-hexenoic acids, most useful for distinction, are tabulated below (Table I).

According to Cason[8], the 9–10 μ region is quite useful for differentiation between the *cis* and *trans* isomers. Freeman[10a], has also pointed out the relatively complex structures of this region in his spectra of the *trans* acids. The *cis* isomer shows a single well-defined band at 9.33 μ, in contrast to the

9) On this subject, refer to Cason (*loc. cit.*) and to his spectral data of the higher homologous members. (*J. Org. Chem.*, **19**, 1836 (1954)).

10) N.K. Freeman, (a) *J. Am. Chem. Soc.*, **75**, 1859 (1953), (b) *ibid*, **74**, 2523 (1952).

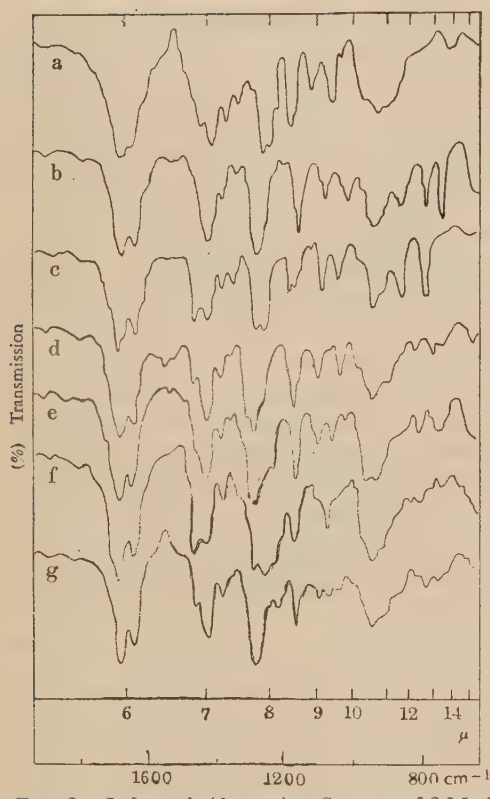

FIG. 2. Infra-red Absorption Spectra of 2-Methyl-2-alkenoic Acids.

(a) αδ-Dimethyl-$\Delta\alpha$-hexenoic acid, (b) Tiglic acid, (c) Angelic acid, (d) *trans*-2-Methyl-2-pentenoic acid, (e) *trans*-2-Methyl-2-hexenoic acid, (f) *cis*-2-Methyl-2-hexenoic acid, (g) *trans*-2-Methyl-2-heptenoic acid. (b-g) J. Cason and M.J. Kalm, *J. Org. Chem.*, **19**, 1947 (1954).

complexity in this region of the *trans* isomers. Of the double band at 6.85, 7.03 μ which is present in all the *trans* acids, the former band is always much weaker and sometimes is only a shoulder, whereas, in the *cis* isomers, the shorter wave-length band proves to be the stronger of the two. As another difference between the two isomers, the overlapping carboxyl bands at 7.8 and 8 μ are indicated. Freeman[10b] indicates that in saturated acids the latter (8 μ) band is always the weaker unless in the presence of an α-methyl substituent, in this case the second being the stronger. The 8 μ band is weaker in *trans* isomers as is seen from Fig. 2 and Table I,

TABLE I

INFRA-RED ABSORPTION SPECTRA OF 2-METHYL-2-ALKENOIC ACIDS

Bands most suitable for differentiation of isomers. (in μ)

αδ-Dimethyl-$\Delta\alpha$-hexenoic acid	*trans*-2-Methyl-2-hexenoic acid*	*cis*-2-Methyl-2-hexenoic acid*
6.10 VS	6.09 VS	6.11 VS
6.85 S	6.85 W	6.84 VS
7.08 VS	7.03 VS	7.04 VS
7.80 VS	7.76 VS	7.78 VS
7.95 Sh	7.95 Sh	8.00 VS
8.18 M	8.15 M	8.12 Sh
8.63 S	8.60 S	8.54 S
9.03 M	9.08 M	9.08 Sh
—	—	9.33 S
9.45 M	9.44 M	—
—	—	9.55 Sh
9.64 W	9.70 W	—
10.7 S	10.7 S	10.55 VS
10.9 Sh	10.9 S	—

Legend to band intensity abbreviations: VS, very strong; S, strong; M, medium; W, weak; Sh, shoulder. * J. Cason, M. J. Kalm, *J. Org. Chem.*, **19**, 1947 (1954)

whilst in the *cis* isomers, the second band is the stronger. Therefore, the relative intensities of the two pairs of bands are reversed in the *cis* and *trans* isomers. The longer wave-length unsaturation bands beyond 11 μ have not been considered for this distinction between *cis* and *trans* acids, because it has been observed by Freeman that these bands are markedly influenced by the molecular environments such as the substituents on or near a double-bond. Recently, Buchta[11] pointed out the 12.3 μ band to be characteristic for "all" *trans*-2, 7-dimethyl-octadiene-2, 6-diacid-1,8 but this seems unsuitable for this distinction. As is apparent from the Table and comparison discussed above, αδ-dimethyl-$\Delta\alpha$-hexenoic acid is concluded to be the *trans* acid.

In addition, the best criterion for identity of the *trans* configuration of this acid is the acid dissociation constant. Steric effects on the acidity of $\alpha\beta$-unsaturated acids have frequently been discussed in the earlier

11) E. Buchta, H. Schlesinger, *Ann.*, **593**, 13 (1956)

literatures. It has been well known that the *cis* isomers of $\alpha\beta$-unsaturated acids are usually stronger than their *trans* isomers. It seems probable that the acid-strengthening effect is due to the twisting of the carboxyl groups away from the plane of the rest of the unsaturated systems, as suggested by Ingold[12]. The pK value of $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid in comparison with those of the isomeric 2-methyl-2-hexenoic acids and of tiglic and angelic acids, are tabulated below.

TABLE II

pK VALUES AT 25°

$\alpha\delta$-Dimethyl-$\Delta\alpha$-hexenoic acid	5.15
Tiglic acid[a]	5.05
Angelic acid[a]	4.30
trans-2-Methyl-2-hexenoic acid[b]	5.13
cis-2-Methyl-2-hexenoic acid[b]	4.44

a) D. H. Hey, *J. Chem. Soc.*, **1928**, 2321.
b) J. Cason, M. J. Kalm, *J. Org. Chem.*, **19**, 1947 (1954).

The ratio of these constants could hardly be influenced significantly by substitution of a larger group* for methyl, and thus pK value (5.15) of $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid unambiguously shows the *trans* configuration.

Therefore, the independent evidence mentioned above leads to the same conclusion. and it follows that the parent $\alpha\delta$-dimethylsorbic acid has the *trans* configuration with respect to $\alpha\beta$-double-bond; thence, *trans* configuration of the side-chain double-bond of both synthetic chrysanthemum-dicarboxylic scids (*trans-trans* m.p. 208°, *cis-trans* m.p. 209°) and of naturally derived chrysanthemum-dicarboxylic acid (*d-trans-trans*, m.p. 163–4°) was experimentally established.

EXPERIMENTAL

All m.p.s and b.p.s were uncorrected.

Ultra-violet light absorptions were determined in *n*-hexane on a Beckman Model-DU quartz spectrophotometer and infra-red absorption spectra were recorded using one-molar solutions in carbon tetrachloride and corbon disulfide on a Perkin Elmer Model-21 double beam recording spectrophotometer. Microanalyses were by the Microanalytical Division, Mitsui Laboratory, Kyoto University.

$\alpha\delta$-Dimethyl-$\Delta\alpha$-hexenoic acid (2,5-Dimethyl-2-hexenoic acid). A 15.0 g quantity of $\alpha\delta$-dimethylsorbic acid[11] (m.p. 134–5°) dissolved in 150 ml of methanol was hydrogenated over Pd-$BaSO_4$ catalyst (1.2 g) in a shaking apparatus until 2564 ml (at 19°) of hydrogen (equivalent to one double-bond) was absorbed. The reduction product thus obtained, was freed from the catalyst and solvent, and carefully distilled under a reduced pressure. The distillate was submitted to partial esterifications in order to eliminate the possible $\Delta\beta$- and $\Delta\gamma$-dihydro acids; the distillate (14.3 g., 95 %) was mixed with 0.2 N-ethanolic hydrogen chloride (70 ml) and kept at room temperature for 5.5 hrs. After the duration, the solution was diluted with four times its bulk of water, made faintly alkaline against lithmus paper by the addition of sodium carbonate. The neutral substance separated was collected with ether, then the aqueous layer was concentrated under a reduced pressure below 50° until free from alcohol. The neutral fraction was again completely removed with ether at this stage. Acidification of the aqueous residue and thorough extraction with ether, followed by drying and removal of the solvent, gave the fraction b.p. 118–122°/10 mm (11.1 g; 73 %). This crop was enough pure at this stage, but in order to obtain the sample of the highest purity for physico-chemical measurements, this was again subjected to partial esterification exactly in the same manner as described above, afforded $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid b.p. 119–122°/10 mm (9.5 g). In further rectifications, only a centre cut of pure $\alpha\delta$-dimethyl-$\Delta\alpha$-hexenoic acid was collected, b.p. 120.5–121.5°/10 mm (6.3 g); n_D^{25} 1.4597; equivalent wt. Found. 141.1 Calcd. for $C_7H_{13}CO_2H$, 142.2; λmax. 218 mμ, ε 14,300; for IR, see Fig. 2-a in the text. It crystallizes in a prism when chilled in dry-ice and melts at about 0°. p-*Phenylphenacylester*, m.p. 54–6° (*Anal.* Found; C, 78.55, H, 7.13, Calcd. for $C_{22}H_{24}O_3$: C, 78.54 H, 7.19). Distillation of the neutral ether extracts combined gave the fraction of ester, b.p. 84–88°/22 mm. n_D^{25} 1.4313 (4.5 g). It decolourizes the permanganate solution at room temperature and absorbs bromine instantly. However, this was not subjected to further investigation.

Ozonization:—One gram of the dihydro-acid (b.p. 120.5–121.5°/10 mm.) in 50 ml of chloroform was treated with an excess of ozone at 0°. The solvent

12) C.K. Ingold, *Structure and mechanism in organic Chemistry*, 743-750 (1953).

* Regardless of the type of branching.

was removed in vacuum, and the remaining ozonide was decomposed with water on a water bath for 10–15 minutes. To this aqueous solution, was then added the solution of 2, 4-dinitrophenylhydrazine in dilute hydrochloric acid to precipitate any carbonyl compound present, and was kept overnight. The yellow precipitate was collected and recrystallized serveral times from ethanol to give 2, 4-dinitrophenylhydrazone of pyruvic acid, m.p. 218° (0.6 g).

The melting point was not depressed by admixture with an authentic specimen. The filtrate from the hydrazone was thoroughly extracted with ether and the ether solution was dried over anhydrous sodium sulphate, and after removal of ether, the residue was distilled to give isovaleric acid (0.3 g), b.p. 170–175°, n_D^{32} 1.4020. p-*Phenylphenacylester* m. p. 77–8° (cf. Drake[13] m.p. 76°). The melting point was not depressed by admixture with an authentic specimen.

Quantitative hydrogenation:—A 0.233 g quantity of the acid (b.p. 120.5–121.5°/10 mm.) in 30 ml of ethanol was hydrogenated over a platinum oxide catalyst (11 mg) in a shaking hydrogenation apparatus, and absorbed 40.0 ml (at 27°) of hydrogen, equivalent to one double-bond. The reduction product was freed from both catalyst and solvent, and distilled to give the fully reduced saturated $\alpha\delta$-dimethylcaproic acid[14] in a yield almost quantitative, b.p. 115–116°/13 mm., n_D^{20} 1.4261. *Amide*, m.p. 102–3°, p-*Phenylphenacylester*, m.p. 66°, identified by mixed melting point comparison with authentic specimens, respectively.

Determination of pK value.—The pK value was determined from the value of the pH at the half neutralisation point in a graph of pH vs. volume of sodium hydroxide solution. A sample of the acid (0.2 g) was dissolved in 30 ml of 50 vol. % methanol-water, then titrated with carbonate-free sodium hydroxide (0.1 N) at a constant temperature (25°). The pH determination was with a Yanagimoto Model-40A pH meter, using glass electrodes; frequent standardizations were run against standard buffer solutions. At least twenty readings were taken after successive addition of sodium hydroxide and after determinations were carried out in triplicate, the values were confirmed to be reliable and reproducible, and corrected for hydrolysis, solvation and repression of the acid[14].

The authors are indebted to Prof. S. Takei for his kind advice and to the Ministry of Education for a Research Grant. Part of this work was carried out during 1952–1953.

13) N.L. Drake, J. Bronitsky, *J. Am. Chem. Soc.*, **52**, 3715 (1930)

14) G.M. Bennett, G.L. Brooks, S. Glasstone, *J. Chem. Soc.*, **1935**, 1821

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 11~17, 1957]

Studies on the Protease of *Pseudomonas*

Part II. Crystallization of the Protease and its Physico-chemical and General Properties

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Received July 30, 1956

The purification of the protease of *Ps. myxogenes* sp., was carried out with the filtrate of a shaking culture of a medium containing glucose of a high concentration, and by application of the following methods, salting out, rivanol treatment, and fractional precipitation with acetone; it was finally succeeded to crystallize the enzyme in a needle-like form obtaining a good yield. This protease preparation was found to reveal homogeneous nature of the electrophoresis at pH 8.07, and further experiments on other physico-chemical and general properties of the preparation were investigated.

INTRODUCTION

In the previous paper[1] of this series, it has been shown that protease production of *Ps. myxogenes* sp. was enhanced with the increasing concentration of glucose of the shaking culture.

The present paper deals with studies on purification and crystallization of the protease from the cultural filtrate of the bacterium. Moreover, the physico-chemical and general properties of the crystalline protease thus obtained, were ascertained.

EXPERIMENTAL AND RESULTS

1) The Method of Isolation of Protease.

Cultivation. *Ps. myxogenes* sp. was cultured in each 100 cc. of a medium containing 7% glucose, 1% $(NH_4)_2HPO_4$, 1% Na_2HPO_4, 0.2% KH_2PO_4, 2% $CaCO_3$, 0.05% $MgSO_4$ and 0.125% C.S.L. (or 0.2% yeast extract) taken in 500-cc Sakaguchi flasks which were placed on a shaker of 7-cm amplitude and 130 r.p.m., and kept at 30°C for 3 to 5 days. When protease activity reached to its maximum (200–250 [u]/ml.[1]), cultivation was immediately stopped.

Purification and Crystallization. 2 M $CaCl_2$ was added to the cultural solution as much until compiete precipitation of calcium phosphate was attained, and then the solution was filtered repeatedly through a layer of Hyflo Super-Cel on a Buchner funnel, until the filtrate became perfectly clear. To 5 liters of the clear filtrate, 2280 g of $(NH_4)_2SO_4$ was added to produce 0.6 saturation. After one day, the precipitate was removed by filtration with filtercel on the paper, and the precipitate dissolved into 50 cc. of 0.01 M $CaCl_2$. Insoluble material was discarded and the clear filtrate dialyzed against 0.002 M $CaCl_2$ solution at 5 to 10°C, for 5 days until NH_4^+ was removed.

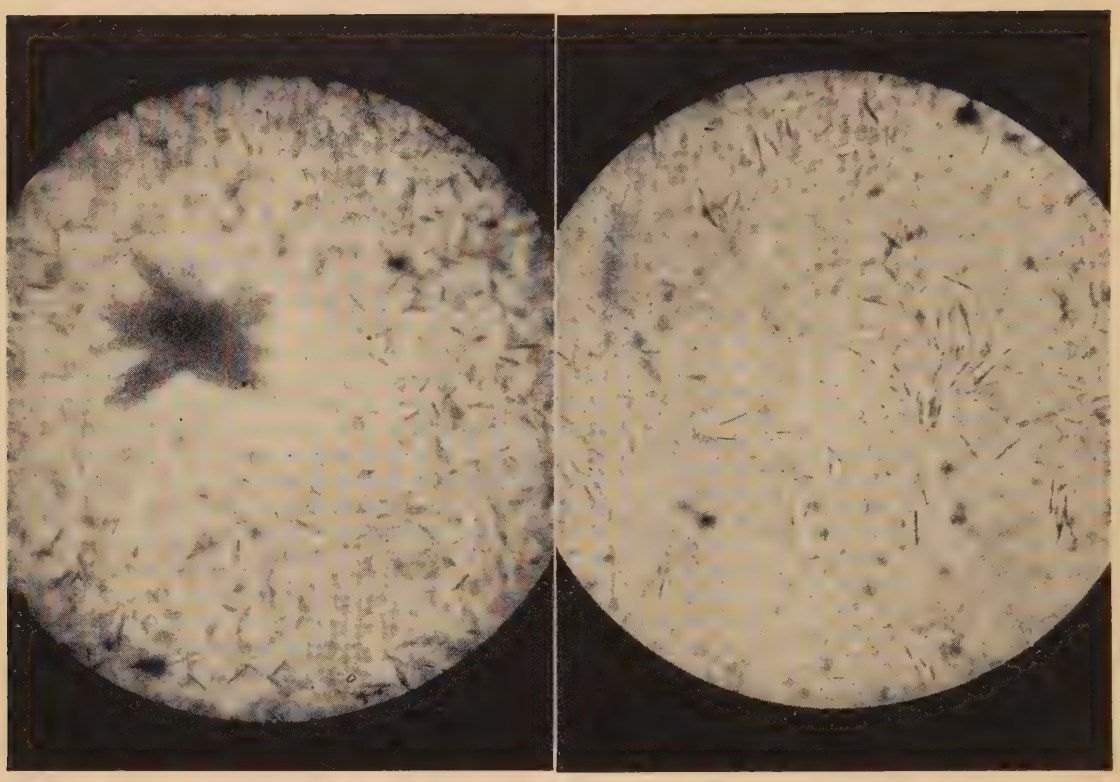
The insoluble material was again discarded, added 1% rivanol solution[2,3,4] to the filtrate until its concentration reached up to 0.025%, then the precipitate was discarded. To the clear filtrate, more rivanol solution was added so as to make the concentration to 0.055%, and the precipitate was collected and then dissolved in 0.5 M ammonium acetate solution. The solution was decolorized with active carbon (0.2 g per 100 cc.). To the decolorized solution, acetone was added under cooling (below 5°C) until the concentration reached up to 65% and the precipitate was collected and then dissolved in 20 cc. of 0.01 M $CaCl_2$ solution. The insoluble material was discarded, and much acetone was added to the clear solution, drop by drop, with stirring so as to get slight turbidity,

1) K. Morihara, This Bulletin, **20**, 243 (1956).

2) Akabori et al., *Proc. Japan Acad.*, **27**, 350 (1951).

3) Akabori et al., *Symposia on enzyme chemistry (Japan)*, **8**, 49 (1952).

4) Miyake et al., *Biochemistry (Japan)*, **26**, 105 (1954).



(A) (× 370) (B) (× 370)

PHOTO 1 Crystalline Protease of *Ps. myxogenes* sp.
(A) crude crystal (B) once recrystallized

then the suspension was stored overnight in a refrigerator. Needle-like crystals appeared. Again, acetone was added to the suspension in the same way (concentration of acetone was about 30–40%, by volume) as mentioned above, and then stored in a refrigerator for more than one day. More than 85% of the protease in the suspension was crystallized in a needle-like form (see Photo. 1 (A)).

After washing the crude crystals with 50% acetone under cooling, recrystallization was succeeded by dissolving the crystals in 20 cc of a 0.01M $CaCl_2$ solution, its pH value adjusted to 9.0 with N-NaOH (as the crystals could not be dissolved in the neutral solution), adjusting the pH to 7.0 with acetic acid, followed by the addition of acetone as mentioned above. Under these conditions, crystallization commenced almost immediately (see Photo. 1 (B)). The crystals were collected and suspended in 20 cc of distilled water. The suspension was frozen and dried under vacuum. The yield obtained was about 500 mg from 5 liters of the cultural solution (about 25% calculated from the total protease activity of the cultural solution).

It is a known fact that crystallization becomes very easy with inoculation of a small amount of the crystals. Even when any initial precipitation by rivanol was not separated, which contained about 40% of the total protease activity, similar crystallization was observed by inoculation of the crystals. The yield of the first crop of crystals was about 900 mg from 5 liters of broth (45% yield from the total activity of the cultural solution).

It is very interesting to note that the material purified by the salting out-procedure had an acylase action on acetyl-DL-methionine, but the crystals obtained here revealed no such action. And the activity of the crystals measured from the liquefaction of gelatin in the usual manner[1], was found to be 500

[u]/mg, showing activity about four times greater than that of the crystalline trypsin (Mochida & Co., Ltd.).

2) Physico-chemical Properties of the Crystalline Protease.

Electrophoresis. Using the Tieselius apparatus, electrophoretic migration at pH 8.07 was attempted with 2.0% solutions of the twice recrystallized protease preparation. The results revealed that there existed only a definite peak which was represented by one homogenous substance, as shown in Photo. 2.

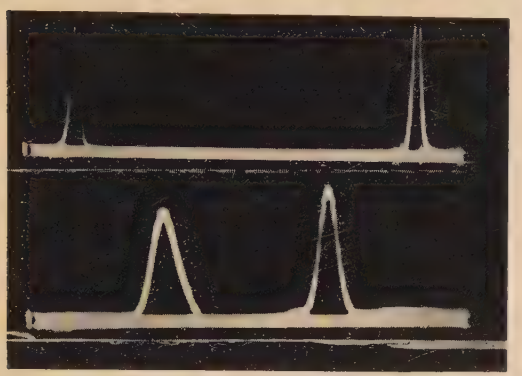


PHOTO 2 Electrophoresis of the Crystalline Protease.
Experiments were carried out with 2% enzyme solution containing phosphate buffer of pH 8.07 under $\mu = 0.1$, 10mA and 61 Volt at 4°C for 2 hours.

Isoelectric point. The paper-electrophoretic pattern of the crystalline protease at various pH adjusted by buffer solution, was studied. The distance of the migration was measured, and the results are shown in Fig. 1. From these results, the isoelectric point was supposed to be pH 5.5–6.0.

Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of the crystalline protease is shown in Fig. 2. The maximum absorption was found at 275 mμ, and the minimum at 250 mμ.

Molecular weight. The molecular weight of the crystalline protease was studied by the diffusion method[5] with the Tieselius apparatus and with an improved model of the Neurath-cell. The results of the diffusion at 30°C are shown in Photo 3.

The diffusion constant ($DA = A^2/4\pi t(Hm)^2 1/G$) was calculated from Photo. 3, and the average value of 9.32×10^{-7}, was obtained. By calculating the molecular weight through the formula ($M = R^3T^3/162\pi^2\eta^3N^2.1/D^3V.1/(f/fo)^3$), substituting the diffusion constant, as-

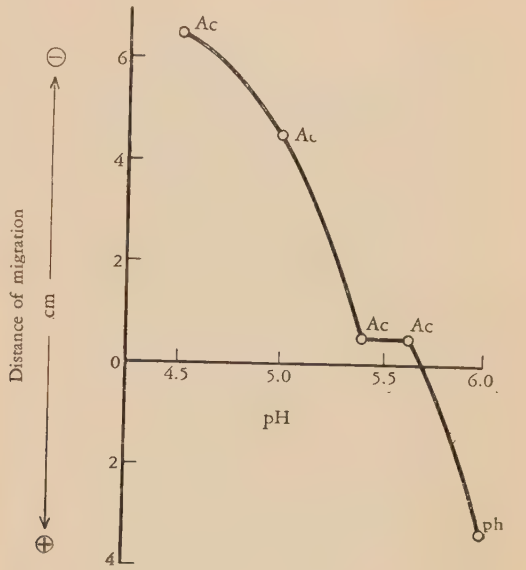


FIG. 1. Paper-electrophoretic Pattern of the Crystalline Protease at Various pH Values of Buffer Solution.
Experiments were carried out with acetate or phosphate buffer of various pH values under $\mu = 0.1$, 1 mA and 500 Volt for 5 hours.
Ac = acetate buffer, Ph = phosphate buffer

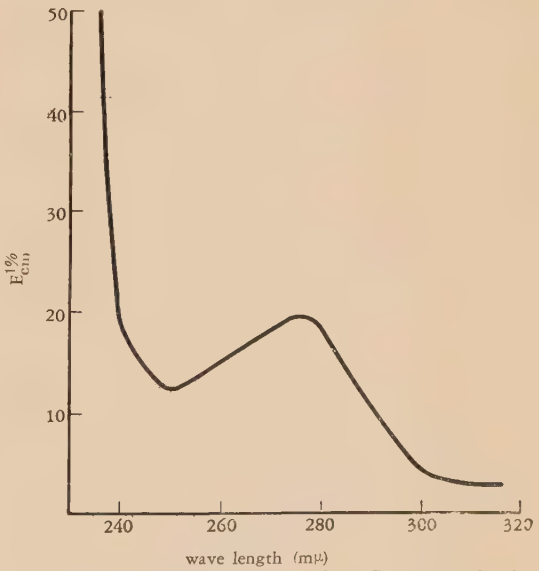


FIG. 2. Ultraviolet Absorption Spectra of the Crystalline Protease.

5) G.L. Miller, K.J.I. Anderson, *J. Biol. Chem.*, **144**, 475 (1952); Watanabe & Iso, *Science (Japan)*, **18**, 421 (1949); Akabori & Mizushima, "*Protein Chemistry*" Vol. **2**, 382 (1954).

suming that the protein was globular in its form, and specific gravity as 1.2, molecular weight was thus decided to be 77,000.

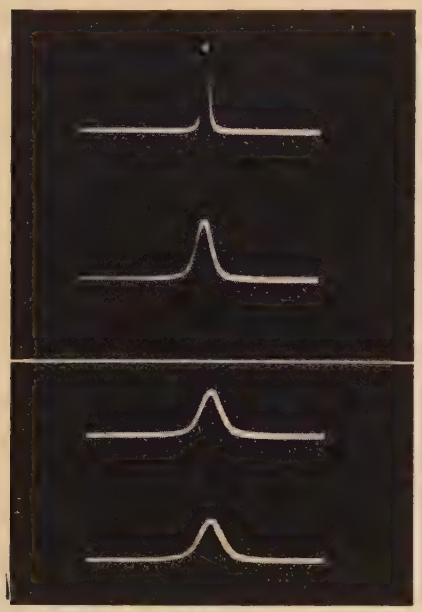


PHOTO 3 Diffusion of the Crystalline Protease.
Enzyme concentration was 1.25% in distilled water adjusted by NaOH to pH 9.6. Temperature was 30°C ±0.001.

Elementary analysis. The composition of the twice recrystallized protease is as follows: C, 46.59%; H, 7.57%; N, 13.56% and ash 2.92%. The majority of ash was supposed to be composed of $CaCl_2$.

Amino acid composition. Crystalline protease 10 mg, in 1 cc. of 6 N HCl was sealed in a test-tube and hydrolyzed at 115°C for 20 hours. The hydrolysate was concentrated under reduced pressure. Examination of amino acids in the concentrated hydrolysate was conducted by paper partition chromatography of two dimensions (Bu-Ac-water and Phenol). From the results, the presence of the following amino acids was confirmed: leucine, isoleucine, valine, tyrosine, alanine, threonine, histidine, glycine, serine, glutamic acid, aspartic acid, and one unknown substance.

Moreover, the examination on the colour reactions of the crystalline protease was made, and the results obtained were as follows: Ninhydrin violet, Biuret red-violet, Xanthoprotein +, Millon +, Adamkiewitz +, Lieberman ± (yellow-violet), Neubauer-Rhode ±, Pauli +, Sakaguchi +, Nitroprusside −. (+ indicates positive, and − negative.) Besides the 11 amino acids shown above, the presence of arginine and tryptophane was also confirmed by examining colour reactions. Also, the absence of S in the crystalline protease was confirmed.

Others. The crystalline protease became water-soluble by treatment of the freeze-drying method but the activity was lowered to 60–70%, while the reason of inactivation was obscure.

3) Stability of the Crystalline Protease.

1. Effect of heat-treatment. For the stock solution of the crystalline protease obtained from *Ps. myxogenes* sp., 2% solution of distilled water was chosen, and when it was employed, it was diluted with water suitably. Of the diluted enzyme solution 0.5 cc was poured into a test-tube containing 4.5 cc of distilled water or 0.01 M $CaCl_2$ solution, previously heated to various temperatures, and the mixed solution was kept at various temperatures for heat-shocking treatment. After 10 minutes, the solution was cooled immediately, and after the remaining protease activity was measured the percentage of inactivation was calculated. The results are shown in Fig. 3.

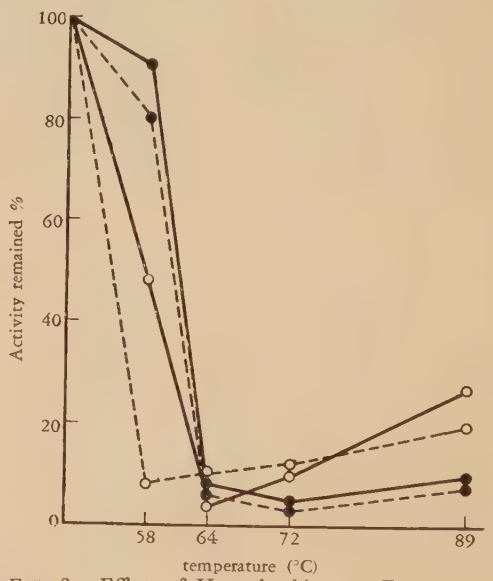


FIG. 3. Effect of Heat-shocking on Enzyme Solution, for 10 min with or without $CaCl_2$.

From Fig. 3, it will be seen that the enzyme is stable up to 50°C in spite of both the presence or

absence of $CaCl_2$, but inactivates markedly at 58°C in distilled water. In case of lower concentrations of the enzyme, further inactivation was observed at this temperature. In a 0.01 M $CaCl_2$ solution, however, the enzyme was almost stable, as the enzyme was protected from heatshocking. At 64°C, the enzyme in both distilled water and 0.01 M $CaCl_2$ solution was inactivated almost completely, showing remaining activity to be below 10%. This shows that the enzyme is not protected by $CaCl_2$ at this temperature. At temperatures excleding 72°C, the enzyme in distilled water was found to be more stable than at 64°C. While, on the other hand, this phenomenon was not observed in the 0.01 M $CaCl_2$ solution. That is to say, the enzyme is protected till the temperature reaches 58°C, but it is rather affected by $CaCl_2$ over 72°C. The above phenomenon seemed curious if inactivation is considered to be caused by denaturation of protein. The author considers this phenomenon as follows:

(1) At 58°C, the instability of the enzyme in distilled water is attributable mostly to autodigestion, according to the theory of Kunitz & Northrop[6]. On the other hand, stability in 0.01 M $CaCl_2$ at this temperature is caused by the formation of a Protease-Ca Complex according to the theory of Green & Neurath[7], and not by auto-digestion.

(2) The instability in either case at 64°C may be caused by auto-digestion. That is, even in the presence of Ca it does not follow the theory of Green & Neurath for high temperature.

(3) At a temperature over 72°C, the reason for instability in distilled water is attributable only to heat-denaturation, and not to auto-digestion. While, on the other hand, in the presence of Ca, the Protease-Ca Complex is partly formed even at this temperature, so that inactivation is caused by auto-digestion and by heat-denaturation as in distilled water. Therefore, it is more unstable in the presence of Ca than in distilled water.

Various inorganic salts were examined in order to investigate whether they showed the same action on the enzyme as $CaCl_2$ or not. The treatment on the enzyme solution was conducted at 58° or 64°C for 10 minutes with various inorganic salts. The results are shown in Table I.

From Table I, it may be concluded that the action of $CaCl_2$ is attributable to the kathion. And it was shown that the action of Ca could be substituted by Mg, Mn and Co.

TABLE I
EFFECT OF HEAT-SHOCKING ON ENZYME SOLUTION WITH VARIOUS KINDS OF SALT

Salt (0.01 M)	Activity remaining	
	58°	64°
KCl	10	13
$MgSO_4$	65	9
NaCl	10	13
$MnSO_4$	77	11
$BaCl_2$	43	7
$ZnSO_4$	22	10
$CdSO_4$	12	11
$NiCl_2$	15	7
$Co(NO_3)_2$	61	13
Na_2SO_4	25	15

The treatment was carried out with 0.001% enzyme solution containing 0.01 M of various salts for 10 minutes.

2. Effect of pH. The diluted solution of the crystalline protease (0.005 or 0.001%) of various pH values, adjusted by NaOH or HCl, was added toluene and stored at 30°C for 24 hours. The remaining activity of the enzyme solution was measured as shown in Fig. 4.

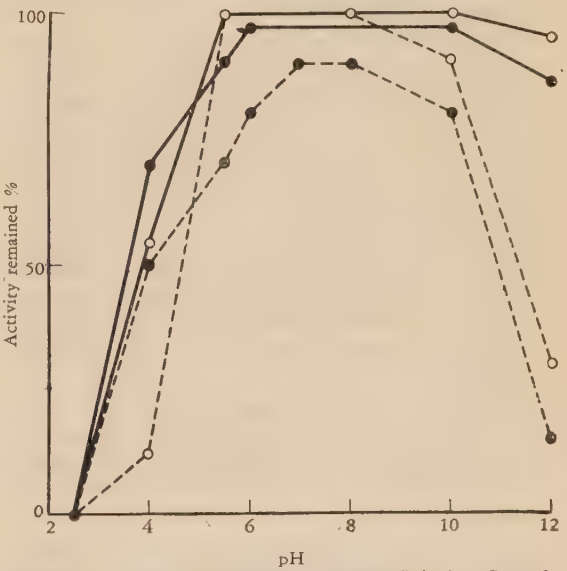


FIG. 4. Effect of pH on Enzyme Solution Stored at 30°C for 24 hrs. with or without $CaCl_2$.
——— enzyme concentration 0.005%
- - - - " 0.001%
● with 0.01 M $CaCl_2$.
○ " distilled water.

6) Kunitz, M., and Northrop, J.H., *J. Gen. Physiol.* **19**, 991 (1936).
7) N.M. Green & H. Neurath, *J. Biol. Chem.*, **204**, 379 (1953).

TABLE II
EFFECT OF METAL ION ON ENZYME SOLUTION

Salts (M)	Activity remaining (%)	Salts (M)	Activity remaining (%)
$AgNO_3$ 4×10^{-3}	66	$MnSO_4$ 10^{-2}	100
$HgCl_2$ 4×10^{-3}	37	$CoSO_4$ 10^{-2}	〃
Pb-acetate 4×10^{-3}	66	$CaCl_2$ 10^{-2}	110
$CuSO_4$ 4×10^{-3}	66	NaCl 10^{-2}	100
$Fe(NH_4)_2(SO_4)_2$ 4×10^{-3}	38	K_2SO_4 10^{-2}	〃
$Fe_2(SO_4)_3$ 4×10^{-3}	19	KF 10^{-2}	〃
$ZnSO_4$ 10^{-2}	72	$MgSO_4$ 10^{-2}	〃

Experiments were carried out using 0.001% enzyme solution containing various kinds of salt at 30 C for 2 hrs.

The enzyme was inactivated completely below pH 2.5 for 30 minutes, and was stable in the range between pH 5.5 to 10.0.

The enzymatic stability was accerelated with the addition of Ca at pH 4.0, but the effect was diminished over pH 5.5.

4) Activation or Inhibition of the Crystalline Protease.

1. Effect of metal ion. Recently, Green et al.[8] have shown that the action of trypsin on benzoyl-L-arginine amide was activated about 25% at its highest, by the addition of Ca, Mn, Cd or Co and inhibited by Hg, Cu, Ag and Zn.

The effect of the various metal ions on the crystalline protease of *Ps. myxogenes* sp. was studied as follows. A 0.001% enzyme solution containing 4×10^{-3} or 10^{-2} M of mineral salt was stored at 30°C for two hours. The remaining activity was measured from the liquefaction of gelatin as usual. The results are shown in Table II.

From Table II, it may be seen that enzymatic action was found to be inhibited by heavy metal ions, such as Fe^{+++}, Fe^{++}, Hg and slightly by Cu, Ag, Pb, Zn. On the contrary, the effect of Ca, Mg, Mn, Co, Na or K was not observed neither as activators nor inhibitors.

2. Effect of reducing agents. It is well known that some proteinases such as papain, ficin or kathepsin are activated by reducing agents, and the classification of proteases was conducted on basis of this significance.

The effect of reducing agents on the crystalline protease was studied. The 0.001% enzyme solution containing various concentrations of HCN or cysteine was stored at room temperature for 2 hours, and remaining activity measured as shown in Fig. 5.

From Fig. 5, it will be seen that at high concentrations, the reducing agents partly inhibited the enzymatic action, but at concentrations of 3×10^{-3} M of HCN or 10^{-4} M of cysteine protease was activated slightly.

3. Others. The effect of phenylhydrazine on the enzyme solution was studied, and no effect was observed on concentrations below 5×10^{-3} M.

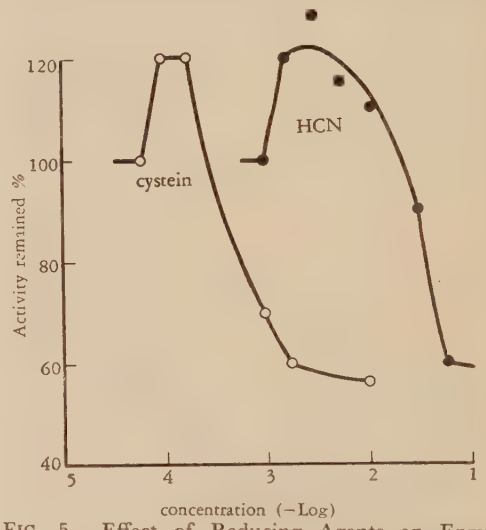


FIG. 5. Effect of Reducing Agents on Enzyme Solution.

Experiment was carried out with 0.001% enzyme solution containing various concentration of HCN (●·—●) or cystein (○—○) stored at room temperature for 2 hours.

Moreover, the activity was not lowered in the case of addition of M/50 EDTA, oxalic or citric acid-ammonium salt by the method of Yamamoto et al.[9]. Therefore, it is concluded that Ca has no relation to

8) Green, N.M., Gladner, J.A., Cunningham, L.M., Jr., and Neurath, H., *J. Am. Chem. Soc.* **74**, 2122 (1952).

9) Yamamoto and Fukumoto, *Symposia on enzyme chemistry (Japan)*, **10**, 32 (1954).

the enzymatic action.

SUMMARY

1) The protease from the cultural filtrate of *Ps. myxogenes* sp. was purified and crystallized in a needle-like form with a high yield (0.5–0.9 g of the once recrystallized ones from 5 liters of broth).

2) The crystalline protease was found to be homogeneous in nature, with electrophoresis at pH 8.07, and the i.e.p. was pH 5.5–6.0. For ultraviolet absorption spectrum, the maximum was observed at 275 mμ and the minimum at 250 mμ. The M.W. has been confirmed to be about 77,000 by the diffusion method. It showed a typical protein reaction in elementary analysis or by composition of amino acids.

3) By heat-treatment, the crystalline protease was found to be stable up to 50°C and was inactivated rapidly over 58°C. At 58°C the enzyme was greatly protected by metal ions such as Ca, Mg, Mn or Co, but these ions did not show any protective action over 64°C. The enzyme was found to be unstable below pH 4.0, but stable between pH 5.5 to 10.0.

4) Enzymatic action of the crystalline protease was inhibited by the heavy metal ions such as Fe^{+++}, Fe^{++}, Hg^{++}, Cu^{++}, Ag^{+}, Pb^{++} or Zn^{++}. The high concentration of HCN, cystein or phenyl-hydrazine lowered the enzymatic action, but it was not affected by the low concentration. Enzymatic activity was not altered by EDTA-treatment, therefore, it is concluded that Ca has no relation to enzymatic activity.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. for his guidance and encouragement throughout this work, and also wishes to thank Mr. E. Masuo of this Laboratory. He is also indebted to Mr. Inoue (for the experiment with the Tieselius' apparatus), Mr. Yoshida (for the paper-electrophoresis), and Mr. Miyahara and Mr. Nakai (for the elementary analysis) of this Laboratory, for their cooperation in carrying out this experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 18~21, 1957]

Enzymatic Resolution of Racemic Amino Acids[1)]

Part IV. Another Purification Method of Mold Acylase and Effect of Metal Ions, Cyanide and EDTA on the Enzyme Activity

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Received August 4, 1956

The mold acylase which catalyzes the hydrolysis of acyl-L-amino acids has been purified previously by the present authors. Another purification method of the enzyme by precipitation as a enzyme-rivanol complex and elution of the enzyme from the complex is presented in this paper.

The effect of metal ions, EDTA (Ethylene-Diamine-Tetra-Acetate) and cyanide on the purified enzyme are also investigated. The enzyme activity is strongly activated by the addition of Co^{++} ion and inhibited by Mn^{++}, Hg^{++} ions and EDTA. The activity inhibited by EDTA is restored to its original level when cobaltous ion is added to the system after contact with EDTA.

Studies on the enzymatic resolution of racemic amino acids have been performed extensively by Greenstein et al.[2,3,4)] They have accomplished the resolution of various amino acids except tyrosine, tryptophan and proline by the use of the optical specificity of a renal acylase on N-acylated-DL-amino acids. Tyrosine and tryptophan were resolved by substituting pancreatic carboxypeptidase for the renal acylase. Similar enzymatic resolution with the mold acylase has also been pursued by the present authors.[5,6,7)] It has been found that the mold acylase is more effective towards the acylderivatives of aromatic-substituted amino acids than towards those of aliphatic amino acids. One of the purification methods of mold acylase has been reported previously[7)]. The present investigation concerns another purification method of the enzyme which depends upon the specific nature of formation of the enzyme-rivanol complex and is more easily applicable for the preparation of the enzyme than those reported earlier, together with the effect of metal ions, cyanide, iodoacetate and EDTA on the hydrolysis of acyl-amino acids by the purified enzyme.

1) This report was presented at the Annual Meeting of Agr. Chem. Soc. Japan, held at the University of Tokyo (March 30, 1956).

2,3,4) J.P. Greenstein et al., *J. Biol. Chem.*, **194**, 455; **198**, 507 (1952); **201**, 847 (1953).

5,6) K. Michi and A. Nonaka, *J. Agr. Chem. Soc. Japan*, **28**, 343; **28**, 346 (1954).

7) K. Michi and H. Nonaka, This Bulletin, **19**, 153 (1955).

EXPERIMENTAL

Purification Method of Mold Acylase. As previously described, one kg of the molded wheat bran which was cultivated under conditions favorable for the production of the acylase was homogenized with distilled water in a mixer. The procedures of concentration of the extract and fractionation with 0.6 saturation of ammonium sulfate under careful adjustment of pH 6.0–7.0 were similar to those reported previously[7)].

The precipitate of the crude enzyme with ammonium sulfate was dissolved in 100 to 200 ml of cold distilled water and the insoluble matter was discarded by centrifugation. The solution was dialyzed overnight against cold distilled water in a refrigerator. The

content of the dialysis sack was treated with 1.2 volumes of chilled acetone in a cold bath and the mixture was allowed to stand overnight in a refrigerator.

The centrifuged precipitates of acetone fractionation were dissolved in 100 ml of cold distilled water and the insoluble matter was removed by centrifugation. The supernatant liquid was treated with 1 ml of 2% aqueous rivanol solution. The acetone treatment can be omitted and the dialyzed solution of ammonium sulfate precipitates treated directly with rivanol solution. The small amount of precipitate which formed was removed by centrifugation and excess 2% rivanol solution was added to the supernatant liquid in order to complete precipitation of the rivanol-enzyme complex. The second precipitate was separated by centrifugation and the supernatant liquid was stored for the amylase test after removal of pigment with acid clay.

The rivanol precipitate was eluted several times with each 10 ml of a 0.5 M McIlvaine buffer (pH 6.2), by stirring. The final-volume of the eluates was about 70 ml and the eluate was treated with 5–7 g of acid clay by shaking for 5 minutes in order to remove the pigment. The eluate, almost colorless, was separated by centrifugation. Then the adsorbate was washed with a small amount of the buffer and the washings were added to the eluate. The enzyme protein was salted out by saturation of ammonium sulfate from the above eluate with caution to pH adjustment in a cold bath.

The purified enzyme solution of the above precipitate contained 100–200 mg of solid matter, and this amount approximates to catalyze 300 g of hydrolysis of acetyl-DL-phenylalanine.

Enzymatic Assay. Various fractions of the purification procedure were tested for their acylase activity. The acylase activity was expressed by the initial hydrolytic rate of acetyl-DL-phenylalanine. Digests were composed of 0.5 ml of 80 mM solution of acetyl-DL-phenylalanine, 1 ml of 0.1 M phosphate buffer (pH 7.8) and 0.5 ml of the acylase solution. After incubation of the digest at 38° for 1 hour, the digest was diluted to 2 mM solution of the susceptible substrate, namely the L-isomer. A 0.2 ml portion of the above solution was treated with the ninhydrin reagent of Stein and Moore, as described previously.[7] The rate of hydrolysis were expressed in terms of micromoles of the liberated L-phenylalanine in the digests.

Since it appeared that the crude enzyme fraction salted out with 0.6 saturation of ammonium sulfate is contaminated with amylase, distribution of the amylase activity was measured at various stages of the purification procedure. Amylase activity was expressed in terms of ml of 1% soluble starch hydrolyzed to give violet with iodine (IKI) solution by the modified Wohlgemuth's method.

RESULTS AND DISCUSSION

Distribution of Acylase and Amylase Activities at Various Fractionation Stages. The results of purification of the mold acylase are summarized in Table I. As shown in Table I, it appears that the entire acylase activity is contained in the second rivanol precipitate, this fraction showing the highest enzyme activity while any other fraction were found to have no activity. Good purification was achieved by elution of the rivanol precipitate

TABLE I

DISTRIBUTION OF ACYLASE AND AMYLASE ACTIVITIES AT VARIOUS FRACTIONATION STAGES IN PURIFICATION PROCEDURE OF MOLD ACYLASE

Purification Procedure	Acylase						Amylase	
	Experiment (a) Activity		Yield	Experiment (b) Activity		Yield	Experiment (b) Activity	Yield
	O.D.	mM	%	O.D.	mM	%	$D_{30}^{38°}$	%
Ppt. with 0.6 saturation of ammonium sulfate	.356×2	3.5	100	.335×2	3.0	100	200	100
Acetone ppt.	.470	1.8	51					
Rivanol 1st ppt.	.00			.00			20	10
Rivanol 2nd ppt.	.300	1.4	40	.270	1.35	45	50	25
Supernatant fluid of 2nd precipitation	.00			.00			90	45
Final ppt. with ammonium sulfate	.230	1.25	36					

with a 0.5 M McIlvaine buffer (pH 6.2) followed by precipitation with ammonium sulfate resulting in an overall yield of 36 percent.

The separation of a contaminated amylase was not satisfactory. Amylase activity was distributed approximately half in the supernatant fluid of the rivanol precipitate and in a lesser degree in each fraction.

Effect of Metal Ions, Cyanide and EDTA. The effect of various metal ions on the acylase activity was studied. The results, which are shown in Figs. 1 and 2, indicate that the

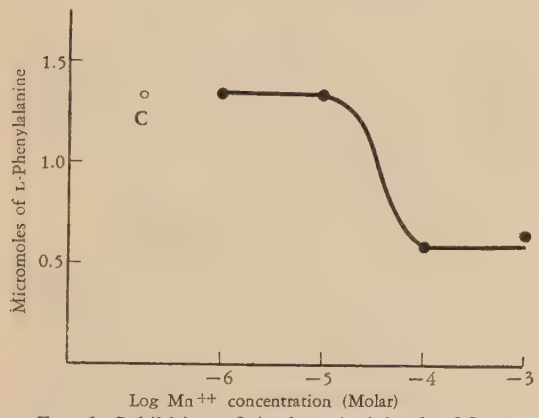

FIG. 1. Inhibition of Acylase Activity by Mn^{++}.
C: None

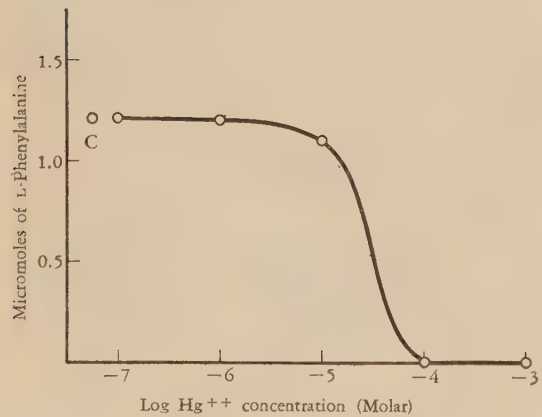

FIG. 2. Inhibition of Acylase Activity by Hg^{++}.

enzyme activity is strongly inhibited by Mn^{++} and Hg^{++}, especially at 10^{-4} molar concentrations. And the addition of Mg^{++} and Zn^{++} ions did not have appreciable influence on enzyme activity.

The effect of the varying concentration of Co^{++} ion on the enzyme activity is shown in Fig. 3. In the presence of Co^{++} ion, the hydrolysis of acetyl-L-phenylalanine was strongly stimulated although concentration higher than 10^{-3} molar showed decreasing activation. Maximal activity was attained at $10^{-4}-10^{-3}$ molar concentration.

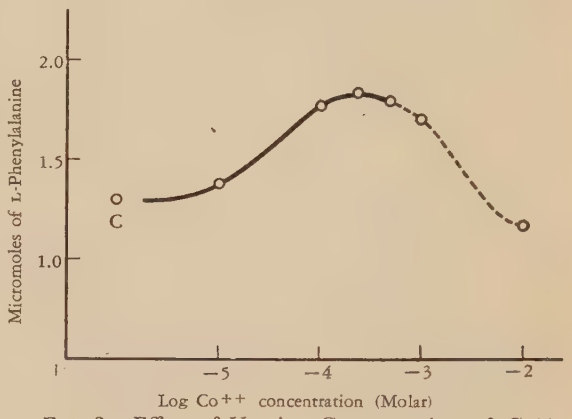

FIG. 3. Effect of Varying Concentration of Co^{++} on Acylase Activity.

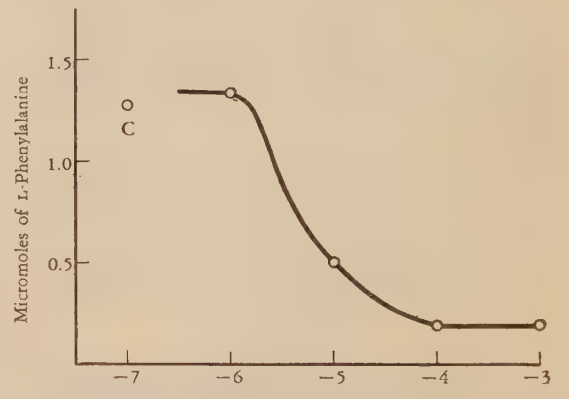

FIG. 4. Inactivation of Acylase by EDTA.

As shown in Fig. 4 in the presence of 10^{-4} molar EDTA, activity of the enzyme was strongly inhibited. However, this effect was reversed by the addition of $10^{-3.7}$ molar Co^{++}. As shown in Table II, the enzyme activity

TABLE II
INACTIVATION OF ACYLASE BY EDTA AND REACTIVATION BY Co^{++} ION

Digests No.	Content	Addition of Co^{++} $10^{-3.7}$ molar (after 0.5 hr's incubation)	Enzyme Activity Incubation period 0.5 hr. O.D.	mM	1 hr. O.D.	mM
1	Sub.+E.	−	0.108	1	0.177	1.15
2	Sub.+E.	+			0.256	1.35
3	Sub.+E.+EDTA	−	0.018	0.35	0.018	0.35
4	Sub.+E.+EDTA	+			0.260	1.36

Digests were composed of 0.5 ml of 80 mM solution of acetyl-DL-phenylalanine, 1 ml of 0.1 M phosphate buffer (pH 7.8) and 0.5 ml of the acylase solution (0.25 mg of purified enzyme) with (No. 3, 4) or without EDTA (No. 1, 2) 10^{-4} molar concentration. After 0.5 hour's incubation at 38° the cobaltous ion was added in a concentration of $10^{-3.7}$ molar to digests Nos. 2 and 4. Enzyme activity was expressed in terms of micromoles of the liberated L-phenylalanine.

was restored to its original level when cobaltous ion was added to the system after contact with EDTA.

The mold acylase resembles other peptidase and the acetylornithinase of *E. coli* which

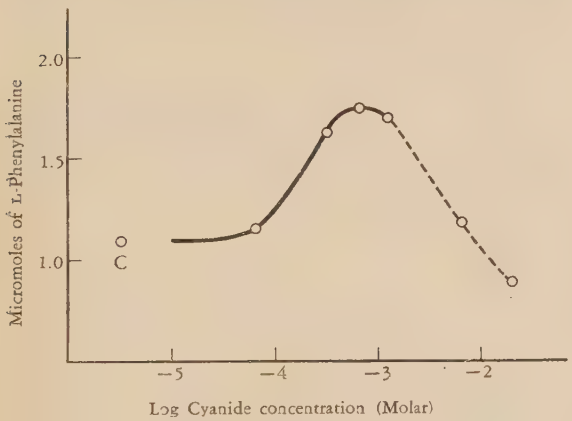

FIG. 5. Effect of Varying Concentration of Cyanide upon Acylase Activity.

has recently[8] been reported in the feature of activation by the cobaltous ion. These results show that the acylase must be a metal enzyme, these findings thus suggesting that Co^{++} ion must be a cofactor.

The effects of iodoacetate and cyanide on the enzyme activity were tested. The addition of iodoacetate failed to inhibit the acylase activity. In the presence of 10^{-3} cyanide, enzyme activity was activated as shown in Fig. 5. The reason of the activation with the cyanide is so far not clear.

SUMMARY

The mold acylase has been purified by precipitation of the enzyme-rivanol complex and elution from the complex, together with fractionation with ammonium sulfate and acetone.

The mold acylase appears to be specifically activated by Co^{++} ion and inactivated by Mn^{++} and Hg^{++} ions as well as by EDTA.

The enzyme activity inhibited by EDTA was restored to its original level with the addition of the cobaltous ion.

This research was aided by a grant from the Ajinomoto Company.

8) H.J. Vogel and D.M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 22~29, 1957]

Syntheses of Pyrethidic* (Chrysanthemumdicarboxylic) Acid

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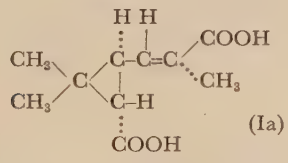
Received August 13, 1956

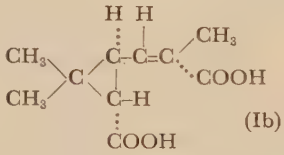
Three racemates of pyrethidic (chrysanthemumdicarboxylic) acid were synthesized by various routes including schemes (2), (3), (4) and (5); thus, the total syntheses of three of the possible four racemates were attained. The stereochemical configurations for these racemates were tentatively assigned although no definite evidence was available. *d-trans*-Pyrethidic acid, naturally occurring chrysanthemumdicarboxylic acid, was also synthesized via scheme (2)

Pyrethidic (chrysanthemumdicarboxylic) acid (I), has been known as the acid component of pyrethrin II and cinerin II which are insecticidal principles occurring in pyrethrum flowers. The skeletal structure (I) has two asymmetric carbon atoms in the cyclopropane ring and one double bond, therefore eight optically active isomers, that is, four racemates can exist. Conventionally, and *dl-cis*-isopyrethidic acid which is isomeric with the natural acid both in double bond and cyclopropane ring; we further tentatively assigned these structures as (Ia), (Ib), (Ic) and (Id), respectively.

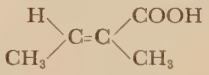
Harper and Sleep[1] synthesized *dl-trans*-pyrethidic acid via Scheme (1). Inouye, Takeshita and Ohno[2] obtained the second racemate *dl-cis*-pyrethidic acid along with



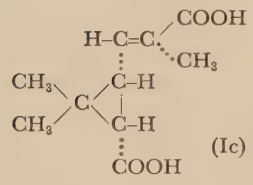
(Ia)



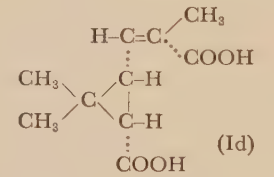
(Ib)



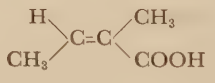
(IV)



(Ic)



(Id)



(V)

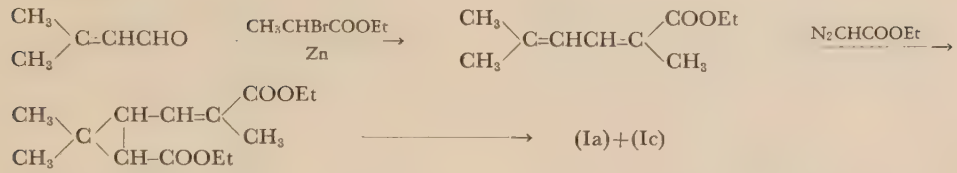
we distinguish them from each other as *dl-trans*-phyrethidic acid which is the racemic form of the natural acid, *dl-trans*-isopyrethidic acid which is the geometric isomer of the double bond, *dl-cis*-pyrethidic acid which is the *cis* modification of the cyclopropane ring, *dl-trans*-isomer by the same route. Although the *cis-trans* relation of the cyclopropane ring was definitely established by ozonolysis and derivation to caronic acid, hitherto, no information was available concerning the double bond.

* Although the name "chrysanthemumdicarboxylic acid" has been commonly accepted, in this paper an alternate name "pyrethidic acid" is designated in place of the foregoing long one.

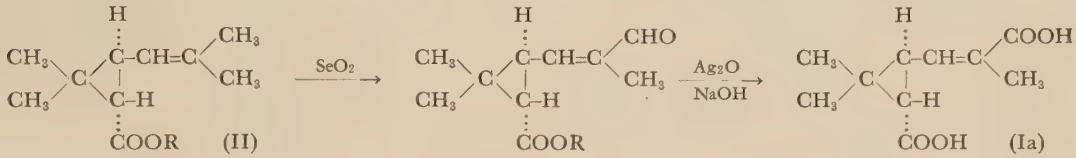
1) S.H. Harper and K.C. Sleep, *Chem. & Ind.* **1954**, 1538.

2) Y. Inouye, Y. Takeshita and M. Ohno, This Bulletin, **19** 193 (1955).

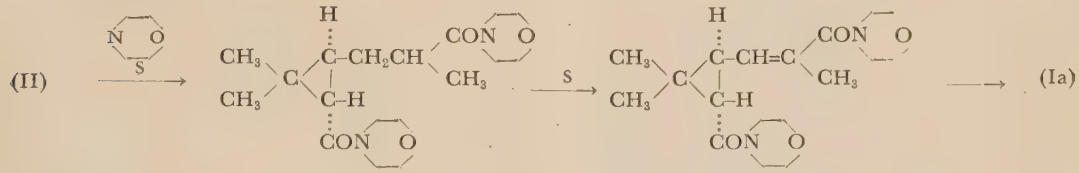
Scheme (I)



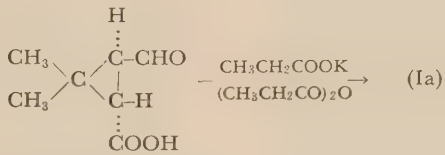
Scheme (2)



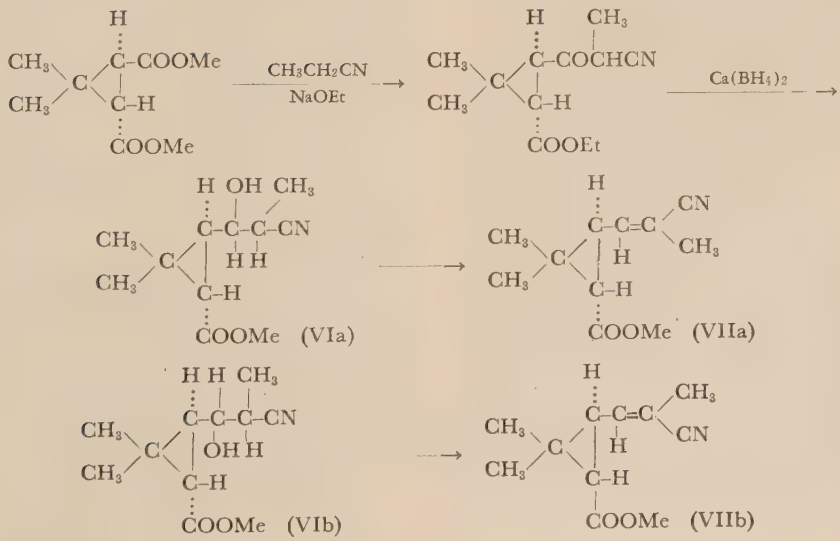
Scheme (3)



Scheme (4)



Scheme (5)



The present authors[3] in a previous paper reported the preparation of *dl-cis*-pyrethidic acid, *dl-trans*-pyrethidic acid, and natural *d-trans*-pyrethidic acid by a new method shown in scheme (2) which is different from that of Harper's.

Now in this communication, we wish to report various syntheses of three out of the four possible racemates including hitherto unknown isomer (Ib), together with more detailed accounts of our preliminary report.

Starting with easily obtainable *dl-trans*-chrysanthemic acid (II), selenium dioxide oxidation in a proper solvent followed by silver oxide oxidation in sodium hydroxide solution, we obtained a racemate (Ia) which obviously had the *trans* cyclopropane ring and possibly the more stable tiglic acid (IV) type double bond, and is certainly identical with Harper and Inouye's racemate although no direct comparison was made. Since two methyl groups of (II) are electronically identical and neither of them are sterically hindered, selenium dioxide would attack both groups in a comparable rate, and the resulting mixed aldehydic acid must have converted into the more stable one in which the carbonyl group is more remote from the carboxyl and cyclopropane ring, consequently, the structure of the final product is probably (Ia).

dl-cis-Chrysanthemic acid afforded another racemate on a sequel of the Scheme (2), which was tentatively assigned to be (Ic). This has clearly *cis* cyclopropane ring and is certainly identical with the acid obtained by Inouye et al., therefore, so far as their starting material, α, δ-dimethylsorbate was homogeneous, this acid should be (Ic). The two possible aldehydic acids in this case have almost the same stability from the point of repulsion between the carbonyl and carboxyl, however, the tiglic type of one is more stable than the other concerning the steric interference between cyclopropane ring and carbonyl. Therefore, (Ic) was expected to and in fact did predominate.

Laborious isolation of the alternate acids (Ib, Id) from the mother liquor was attempted, however, the only identifiable material was caronic acid.

A similar reaction starting with *d-trans*-chrysanthemic acid gave *d-trans*-pyrethidic acid which is identical in all respects with the natural acid. Since the absolute configuration of the former was established[4], this result indicates the absolute configuration of the latter to be (Ia).

A modified Willgerodt reaction of ethyl chrysanthemate (*cis*, *trans* mixture was used) should, we expected, lead to dihydropyrethidic acid, however, it is further oxidized in ordinary condition of the reaction to afford *dl-trans*-pyrethidic acid which was the only product isolated in the pure state. The identity was established by mixed melting point and infrared spectrum. The formation of the more stable (Ia) is reasonably understood and also support the correctness of the stereochemical assignment.

The third route is shown in Scheme (4) in which *dl-trans*-caronaldehydic acid underwent Perkin reaction and afforded a moderate yield of *dl-trans*-pyrethidic acid. This fact together with the foregoing discussions strongly support the correctness of the structure (Ia), because the more stable tiglic type acid should be formed in the ordinary Perkin reaction, as for example, acetaldehyde was reported[5] to give tiglic acid (IV) itself when treated with potassium propionate and propionic anhydride.

The route of the fourth synthesis was shown in Scheme (5). *dl-trans*-Caronylpropionitrile which was obtained by condensation of dimethyl caronate and propionitrile in the presence of sodium ethoxide was reduced with calcium borohydride[6] in cold methanol

3) M. Matsui, M. Miyano and K. Yamashita, *Proc. Japan Academy* **32**, 353 (1956).

4) L. Crombie and S.H. Harper, *J. Chem. Soc.*, **1954**, 470.

5) F. Kietreiber, *Monatsh.*, **19**, 735 (1898).

6) M. Matsui and M. Miyano, This Bulletin, **20**, 139 (1956).

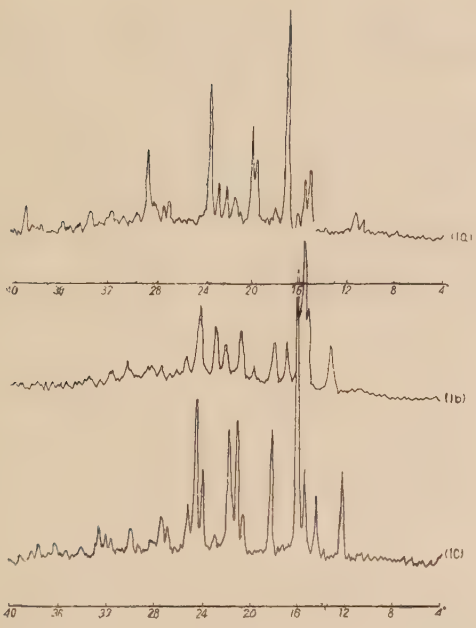


FIG. 1. X-Ray Diffraction Pattern of (Ia), (Ib) and (Ic).

Tube Rating 30 KVP 15MA, Divergence Slit 1° Receiving Slit 0.006", Scatter Slit 1°, Scale Factor 16, Multiplier 1 Time Constant 2 sec.

Norelco X-Ray Diffractometer and Kα-ray from copper anticathode was used. Kβ-Ray was completely omitted by the nickel filter.

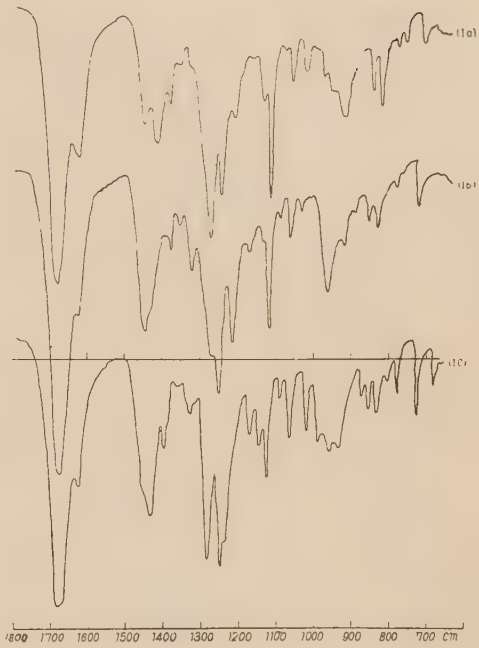


FIG. 2. Infrared Spectrum of (Ia), (Ib) and (Ic).

Perkin-Ermer Infrared Spectrophotometer was used. The sample was powdered with potassium chloride and pressed between potassium bromide discs.

and the resulting hydroxynitrile was dehydrated by distillation in the presence of *p*-toluenesulfonic acid and finally saponified by alkali. The product was clearly distinguished from (Ia), (Ic) by X-ray diffraction pattern (Fig. 1), mixed melting point and infrared spectrum (Fig. 2). Since the acid has clearly the *trans*-cyclopropane structure and is yet different from (Ia), it must be (Ib). Although the explanation of this result is somewhat ambiguous, the most reasonable one may be as follows. The reduction with borohydride afforded thermodynamically the more stable (VIa) rather than (VIb)[7] (this assumption is true so far as –CN is sterically smaller than $-CH_3$ group.) and the former then afforded (VIIa) rather than (VIIb) upon dehydration by *trans* elimination of water.

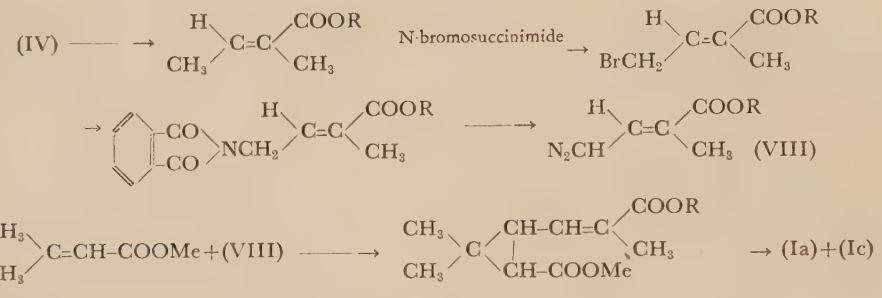
A serious objection is that no conversion of (Ib) into (Ia) was occurred during alkaline saponification of ester group in spite of the fact that under similar conditions angelic acid (V) is almost completely isomerized to tiglic acid. Isomerization did, we presume, in fact occurr to some extent. X-Ray diffraction pattern of the acid showed it to have a somewhat heterogeneous crystalline structure (Fig. 1), although elaborated isolation of isomerized (Ia) was failed.

Analogous schemes including (a) condensation of caronic halfester-chloride with ethyl sodiomethylmalonate, followed by sodium borohydride reduction and decarboxylation, (b) condensation of the half-ester chloride with ethyl sodioacetopropionate and subsequent acidic cleavage and sodium borohydride reduction and dehydration etc. failed.

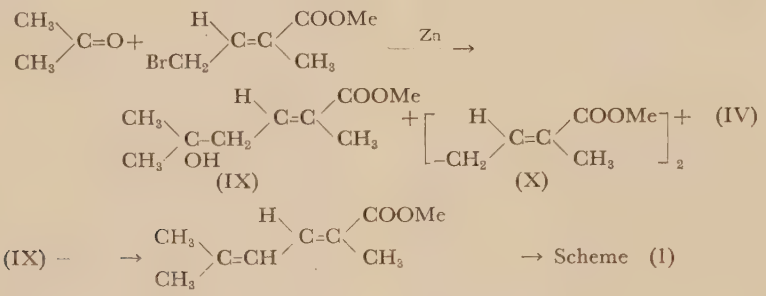
With all these evidences, we believe (Ia), (Ib) and (Ic) are the correct structures of the three acids although no direct evidence

7) W.G. Dauben, G. J. Fonken and D. S. Noyce, *J. Am. Chem. Soc.*, **78**, 2579 (1956).

Scheme (6)



Scheme (7)



was available.

Two unambiguous syntheses shown in Schemes (6) and (7) starting from tiglic acid were subsequently undertaken.

The route of Scheme (6) was unsuccessful because of the extreme lability of the intermediate diazoester (VIII).

The hydroxyester (IX) was obtained as a minor product together with dicarboxylic acid ester (X) and the reduced methyl tiglate (main product), however, dehydration (phosphorus pentoxide in benzene, acetyl chloride in collidine, phosphorus oxychloride in pyridine, or distillation in the presence of *p*-toluenesulfonic acid) and saponification of (IX) afforded no crystalline product, which has thus so far been unsuccessful.

Acknowledgement. The authors should like to express their thanks and appreciation to Dr. K. Ohsaki of Osaka University for the interpretation of Norelco X-ray diffractometry, and to Mr. Y. Ohsumi and Mr. S. Morita of the Mitsubishikasei Co., Ltd. for the infrared spectroscopy.

Sincere thanks are also offered to Professor R. Yamamoto and Professor Y. Sumiki for their interest in this work.

Microanalytical data are owe to the members of The Analytical Laboratory of the Department of Agricultural Chemistry, University of Tokyo, to whom the authors are grateful.

They are also indebted to the Sumitomo Chemical Co. Ltd. and the Daidô Jochûgiku Co. Ltd. for the generous gifts of the materials for this study.

EXPERIMENTAL

***dl-trans*-Pyrethidic Acid (Ia)** (*A*). *Via Scheme* (*2*): Methyl *trans*-chrysanthemate (10 g)[8] in 50 ml of dioxane was refluxed for one hour with 7.3 g of selenium dioxide. Separation of the solution from precipitated selenium, distillation, and redistillation afforded the desired aldehydic ester (6.5 g) boiling at 135–145°/11 mm, n_D^{30} 1.4956; the middle distillate is analysed as C, 57.1; H, 8.1%, calculated for $C_{11}H_{16}O_3$ requires C, 57.3; H, 8.1%. 2, 4-Dinitro-

8) I.G.M. Campbell and S.H. Harper, *J. Chem. Soc.*, **1945**, 285 M. Matsui and M. Miyano, This Bulletin **19**, 150 (1955).

phenylhydrazone, recrystallized from alcohol, formed scarlet needles, m.p. 157°. *Analysis;* Found: C, 54.1; H, 5.5; N, 15.1%. Calculated for $C_{18}H_{22}O_6N_4$ requires: C, 54.3; H, 5.3, N, 14.9%.

The aldehydic ester (6 g) was stirred for 30 min at 70° in 150 ml of 4% aqueous sodium hydroxide solution with silver oxide which had been freshly prepared from 11 g of silver nitrate and sodium hydroxide solution. Separation from silver and acidification with sulfuric acid afforded *dl-trans*-pyrethidic acid which precipitated in the aqueous solution. Pure *dl-trans*-pyrethidic acid (3.5 g) melting at 200°, was obtained by recrystallization from aqueous ethyl alcohol using active carbon. *Analysis*, Found: C, 60.8; H, 6.9%. Calculated for $C_{10}H_{14}O_4$ requires: C, 60.6; H, 7.1%. By ozonolysis, *dl-trans*-caronic acid, m.p. 212°, was obtained. Some less pure *dl-trans*-pyrethidic acid (0.9 g) was obtained from the mother liquor by ether extraction.

(*B*). *Via Scheme* (*3*): A mixture of ethyl chrysanthemate (50 g, the technical product of *cis, trans* mixture was used), commercial morpholine (50 g) and flower of sulfur (11 g) was heated under gentle reflux on an oil bath (150°) for 10 hrs. Sodium hydroxide (30 g) in water (150 ml) was then added and heated under reflux for 6 hrs. Water (100 ml) was added to the reaction mixture from which acidic fraction was extracted with ether by percolation in the usual manner. The ether extract was dried over sodium sulfate, concentrated and steam distilled until opalescence was completely disappeared (about 10 l of the distillate was obtained). The residue was concentrated and dissolved in methanol, treated with active carbon, concentrated under diminished pressure, esterified with diazomethane in the ordinary way. The methyl ester was distilled and the fraction which boiled at 145°/12 mm was collected (5.0 g) had n_D^{17} 1.4840. *Analysis*, Found: C, 63.0; H, 8.2%. Calculated for dimethyl pyrethidate $C_{12}H_{18}O_4$ requires: C, 63.7; H, 8.0%. It was saponified in methanolic sodium hydroxide under reflux for 6 hrs. The solvent was removed and acidified with dilute hydrochloric acid. The crystals thus obtained, were dried on a porcelain plate and recrystallized from hot water. The m.p. and the mixed m.p. with authentic *dl-trans* pyrethidic acid was 195–6°. The identity was also confirmed by infrared spectrum. *Analysis*, Found: C, 61.0; H, 7.5%. Calculated for $C_{10}H_{14}O_4$ requires: C, 60.6; H, 7.1%.

(*C*) *Via Scheme* (*4*): Caronaldehydic acid[9] (1.0 g) and freshly fused potassium propionate (5 g) in propionic anhydride (10 ml) were heated to 170° for 5 hrs. The mixture was poured into water (150ml), boiled for 20 min., treated with active carbon and filtered. The filtrate was concentrated under diminished pressure, acidified with a small amount of hydrochloric acid, and set aside in a refrigerator overnight. The crude crystalline needles, thus obtained (200 mg., m.p. 190–196°) were recrystallized from hot water. The m.p. and the mixed m.p. was 198°. *Analysis*, Found: C, 61.4; H, 6.9. Calculated for $C_{10}H_{14}O_4$ requires: C, 60.6; H, 7.1%.

***dl-cis*-Pyrethidic Acid (Ic)** Methyl *cis*-chrysanthemate (10 g) in 50 ml of dioxane was refluxed for one hour with 9 g of selenium dioxide. Separation of solution from precipitated selenium and distillation and redistillation afforded 6 g of the desired aldehydic ester boiling at 140–145°/17 mm, n_D^{31} 1.4994. *Analysis*, Found: C, 66.8; H, 8.4%. Calculated for $C_{11}H_{16}O_3$ requires: C, 67.3; H, 8.1%. 2, 4-Dinitrophenylhydrazone was prepared and recrystallized from ethyl alcohol, forming scarlet crystals, of m.p. 192°. *Analysis:* C, 54.0; H, 5.5; N, 15.0%. Calculated for $C_{17}H_{20}O_6N_4$ requires C, 54.2; H, 5.3; N, 14.9%.

The aldehydic ester (5 g) was stirred for 30 min. at 90° in 140 ml of 5% sodium hydroxide solution with silver oxide which was freshly prepared from 9.5 g of silver nitrate and sodium hydroxide solution. The solution was separated from silver, acidified with 40 g of 25% sulfuric acid and left standing in a refrigerator. *dl-cis*-Pyrethidic acid which precipitated was collected and washed with water. Recrystallization from aqueous ethyl alcohol using a small amount of active carbon, afforded 3.5 g of colorless *dl-cis*-pyrethidic acid melting at 204°. *Analysis*, Found: C, 60.9; H, 7.2%. Calculated for $C_{10}H_{14}O_4$ requires: C, 60.6; H, 7.1%. By ozonolysis *dl-cis*-caronic acid, m.p. 175°, was obtained. Additional *dl-cis*-pyrethidic acid, less pure in quality, was obtained by concentration of the mother liquor.

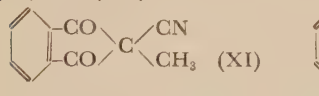
***d-trans*-Pyrethidic Acid (Ia)** Methyl *d-trans*-chrysanthemate (17 g), which was obtained from free acid[10] and diazomethane, was refluxed for one hour in 50 ml of dioxane with 10 g of selenium dioxide. The subsequent procedures, which were identical with those described above, afforded 3.5 g of recovered starting ester boiling at 85–130°/10 mm and 10 g of aldehydic ester boiling at 135–145°/11 mm, n_D^{19} 1.4970.

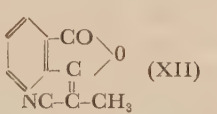
9) M. Matsui et al., This Bulletin, **20**, 89 (1956).

10) I.G.M. Campbell and S.H. Harper, *J. Chem. Soc.*, **1945**, 285.

Analysis, Found: C, 67.1; H, 7.6%. Calculated for $C_{11}H_{16}O_3$ requires: C, 67.3; H, 8.1%. 2,4-Dinitrophenylhydrazone was produced as scarlet needles, and melted at 116° after recrystallization from alcohol. *Analysis*, Found: C, 54.0; H, 5.6; N, 14.9%. Calculated for $C_{17}H_{20}O_6N_4$ requires: C, 54.2; H, 5.3; N, 14.9%. The aldehydic ester (3 g) was oxidized in 100 ml of 6% aqueous sodium hydroxide solution with silver oxide which was freshly prepared from 6 g of silver nitrate under the same condition as described for the preparation of *dl-trans*-pyrethidic acid, *d-trans*-pyrethidic acid was obtained as colorless needles (1.2 g) after recrystallization from water, m.p. 163–164°, $[\alpha]_D^{19}$ +72.0, (c, 1.987 in methyl alcohol); it was found to be identical with the natural acid by mixed melting point determination.

***dl-trans*-Isopyrethidic Acid (Ib)** (*A*). *A Preliminary Experiment:* Phthaloyl Propionitrile—Sodium ethoxide (from 4.5 g of sodium), diethyl phthalate (44 g, 1/5 mol) and propionitrile (11 g, 1/5 mol) were heated for 15 hrs. on an oil bath (120°). The resulting jerry mixture was dissolved in cold water, acidified with acetic acid, extracted with ether. The ether extract was washed thoroughly with sodium bicarbonate solution and water, dried over calcium chloride, concentrated under diminished pressure (210°/10 mm). The concentrate which readily crystallized on cooling afforded two crystals on recrystallization from benzene—petroleum. The main product melted at 141° (12 g) and the minor product which was slightly soluble in benzene melted at 226°(0.5 g). The mother liquor afforded crystals which melted at 141°(15 g) on distillation. As both crystals showed almost the same analytical value, thus, they may be (XI) and (XII).


(XI)


(XII)

Analysis, Found: C, 71.2; H, 3.8; N, 7.4% (141°)
C, 72.0; H, 3.9; N, 7.5% (226°)
Calculated for
$C_{11}H_7O_2N$; C, 71.4; H, 3.8; N, 7.6%

(*B*). dl-trans-*isopyrethidic Acid* Sodium ethoxide (from 1.76 g of sodium), diethyl *dl-trans*-caronate (14.2 g) and propionitrile (4.2 g) were heated on an oil bath (110–120°) for 10 hrs. The reaction mixture was fully shaken in a mixture of ether, ice and hydrochloric acid. The organic layer was washed with sodium bicarbonate solution and water, dried over calcium chloride, and concentrated. The resulting syrup (8.7 g, which contains about 50% of the desired ketonitrile from the nitrogen analysis) was reduced with calcium borohydride (from 1 g of sodium borohydride) in methanol for 3 days at 15°. Methanol was removed in vacuo from the reduction mixture and sodium bisulfate solution was added. Ether extraction by percolation followed by evaporation of the solvent afforded a thick syrup which was distilled in the presence of *p*-toluenesulfonic acid. The fraction which distilled over 110°/12 mm was collected (6.0 g), consisted of a major amount of caronic ester and a minor amount of the desired unsaturated nitrile. A part (3.5 g) of the syrup was saponified with 30% sodium hydroxide solution (5 g sodium hydroxide was used) under reflux for 5 hrs. Continuous ether extraction of the acidic material in the usual manner, and evaporation of the solvent afforded the first crystals which melted at 211° and was not depressed on admixture with authentic *trans*-caronic acid. The second crystals which appeared when the concentrated mother liquor was set aside melted at about 170° and consisted of a comparable amount of the desired *dl-trans*-isopyrethidic acid and inorganic boron compound. On recrystallization from hot water, the pure material was obtained, melted at 175–7°, depressed on admixture with *dl-trans*- or *dl-cis*-pyrethidic acid. *Analysis*, Found: C, 60.8; H, 6.53. Calculated for $C_{10}H_{14}O_4$ requires: C, 60.6; H, 7.1%.

Reduction with sodium borohydride in place of calcium borohydride afforded no identifiable material except caronic acid, possibly because of acidic cleavage of the labile ketonitrile.

Ethyl ω-Bromotiglate Ethyl tiglate (120 g) was heated with N-bromosuccinimide (40 g) until the reaction initiated, after the violent reaction subsided the second portion of N-bromosuccinimide (40 g) was added and heated to react, and the reaction was repeated once more with the third portion of N-bromosuccinimide. Carbon tetrachloride was added and the mixture was refluxed for an hour, cooled, then washed with water and dried with calcium chloride. The fraction boiled at 120°/20 mm was collected which weighed 42 g and had n_D^{31} 1.4863. *Analysis*, Found: Br 38.9%. Calculated for $C_7H_{11}O_2$ Br requires: Br 38.7%.

There is no difinite evidence that bromine is attached to the ω-position, as it was deduced from the theoretical basis and comparison with the similar preparation of ethyl ω-bromocrotonate.

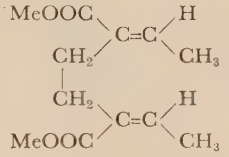
Ethyl Phthaloylaminotiglate Ethyl ω-bromotiglate (20.6 g) and potassium phthalimide (18.6 g) in dimethylformamide was heated on a boiling steam

bath for an hour. The reaction occurred almost spontaneously. Ethyl acetate (50 ml) was added and the mixture was filtered to remove potassium bromide. The filtrate was concentrated in vacuo and cooled to room temperature. The crystals were filtered and washed with petroleum ether, weighed 11.6 g, melted at 168°. *Analysis*, Found: C, 65.2; H, 5.5; N, 5.1%. Calculated for $C_{15}H_{15}O_4N$ requires: C, 65.9; H, 5.5; N, 5.2%.

Reformatski Reaction of Acetone and Methyl ω-Bromotiglate To a mixture of acetone (80 g) and methyl ω-bromotiglate (prepared in an analogous manner to ethyl ester described above) (78 g) in benzene (50 ml) was added zinc fillings (23 g, containing 10% copper). After a violent exothermal reaction had subsided, the mixture was refluxed for an additional one and half hrs. Benzene (200 ml) was added to the cooled mixture and it was washed twice with dilute hydrochloric acid once with water, and dried over calcium chloride. Upon distillation, three fractions were obtained:

134–6°/760 mm	n_D^{24} 1.4300	15 g
120°/20 mm	n_D^{20} 1.4608	3.8 g
165°/20 mm	n_D^{20} 1.4766	3.0 g

The first fraction was reduced methyl tiglate, the second was methyl δ-hydroxy-α,δ-dimethylhexen-2-oate (IX). *Analysis*, Found: C, 63.7; H, 7.6%. Calculated for $C_9H_{16}O_3$ requires: C, 62.8; H, 9.4%. The third fraction was the ester (X). *Analysis*, Found: C, 63.5; H, 7.4%. Calculated for $C_{12}H_{18}O_4$ requires; C, 63.7; H, 8.0%. The last ester (X) was saponified, acidified, and extracted with ether. On evaporation of the solvent, crystals were obtained which melted at 257–8° after recrystallization from aqueous ethanol. The high melting point indicates its symmetry, hence the correct structure of (X). There might be some possibility that the structure of the ester is (XIII) instead of (X).



(XIII)

Attempted dehydration of the second ester with phosphorus pentoxide in benzene, phosphorus oxychloride in pyridine, acetyl chloride in collidine, distillation in the presence of *p*-toluenesulfonic acid and subsequent saponification did not afford any crystalline material.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 30~37, 1957]

Studies on Oxidative Fermentation

Part XV. On the Chemical Pathway involved in the Formation of γ-Pyrone Derivatives from Glucose by *Gluconoacetobacter liquefaciens*.

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Received August 22, 1956

The three γ-pyrone derivatives, i.e. comenic acid, rubiginic acid and rubiginol were confirmed to be formed from glucose via gluconate, 2-ketogluconate and 2,5-diketogluconate by *Gluconoacetobacter liq.*. Other organic acids, such as glycolic and tartronic acids were also formed from glucose via 2,5-diketogluconate. Thus, the presumable pathway of glucose degradation to the three γ-pyrone derivatives is proposed here.

INTRODUCTION

In the previous paper, the authors have reported on the oxidative activities of *Gluconoacetobacter liq.*, which oxidated sugar incompletly and formed a reddish brown pigment in the culture medium containing glucose. Besides aldehyde, formic, acetic, glycolic, tartronic acids, an unknown reducing acid and three substances which gave positive ferric chloride reaction were found as the oxidative products in the culture liquid of 0.8% yeast-extract water, containing 9.30% glucose and 2.5% calcium carbonate after 10 days' shaking culture at 30°C[1,2]. Particular importance was attached to the latter three substances of which one of them was identified as comenic acid[3], while the other two substances were found to be new γ-pyrone derivatives, which were named rubiginol and rubiginic acid. Their chemical structure was studied and they were concluded to be 3,5-dihydroxy-1,4-pyrone and 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid, respectively[3,4,5]. As the formation of γ-pyrone derivatives by microorganism is very interesting from the viewpoint of the microbial sugar metabolism, detailed studies in concern with this problem have been conducted.

EXPERIMENTAL

I. Formation of γ-Pyrone Derivatives from Various Carbon Sources. *Gluconoacetobacter liq.* which was isolated by T. Asai[6] was used throughout this experiment. Ten ml portions of the medium composed of 0.8% yeast extract water and 5% carbon source were dispensed in inverse T-type shaking tubes. Glyceraldehyde, oxalacetate and *cis*-aconitate were fermented on a rather smaller scale, i.e. 1 ml of the medium in a test tube. Organic acids were used as sodium salts, and in the case of other carbohydrates, sterilized calcium carbonate equivalent to one-fourth of the carbon sources was added to each shaking tube just before inoculation. Inoculum was prepared by growing *Gluconoacetobacter liq.* on the koji agar-slant containing calcium carbonate for 3 days at 30°C. These cultures were incubated on a reciprocating shaker at the rate of 117 r.p.m., for 10 days at 30°C. We examined the formation of γ-pyrone derivatives from thirty kinds of carbohydrates and fifteen kinds of organic acids, which cover C_1, C_2, C_3, C_4, C_5, C_6, C_{12}, C_{13}, C_{18} compounds and three polysaccharides. Bacterial growth, pH, color and ferric chloride reaction of the broth are shown in Tables I and II. Among these substrates, positive ferric chloride reac-

1) K. Aida, T. Kojima and T. Asai, *J. Agr. Chem. Soc. Japan* **28**, 517 (1954).
2) K. Aida, T. Kojima and T. Asai, *J. Gen. Appl. Microbiol.*, **1**, 18 (1955).
3) K. Aida, This Bulletin, **19**, 97 (1955).
4) K. Aida, *J. Agr. Chem. Soc. Japan*, **28**, 523 (1954).
5) K. Aida, *J. Gen. Appl. Microbiol.*, **1**, 30 (1955).
6) T. Asai, *J. Agr. Chem. Soc. Japan*, **11**, 610 (1935).

Table I
Formation of γ-Pyrone Derivatives from Various Carbohydrates by *Gluconoacetobacter liq.*

Cn	C-source	growth	pH	color	$FeCl_3$
C_1	methanol	−	6.0		−
C_2	ethanol	−	6.0		−
C_3	glycerol	##	5.8	Y	−
〃	glyceraldehyde	+	5.8	YB	−
〃	dioxyacetone	+	5.6	B	−
C_4	erythritol	##	5.4	YB	−
C_5	L-arabinose	##	5.0	PY	−
〃	D-xylose	##	5.0	PY	−
〃	adonitol	+	5.8	Y	−
C_6	D-glucose	##	5.6	RB	##
〃	D-fructose	##	5.8	PY	+
〃	L-sorbose	−	6.0		−
〃	D-mannose	##	5.0	Y	−
〃	D-galactose	##	5.6	RB	−
〃	L-rhamnose	−	6.0		−
〃	D-sorbitol	±	6.0		−
〃	D-mannitol	##	5.8	PY	+
〃	inositol	−	6.0		−
〃	dulcitol	−	6.0		−
C_{12}	sucrose	±	6.0		−
〃	maltose	+	5.8	PY	−
〃	lactose	−	6.0		−
〃	trehalose	±	5.8		−
〃	cellobiose	##	5.6	Y	−
〃	melibiose	+	5.6	Y	−
C_{13}	salicin	##	5.6	Y	−
C_{18}	raffinose	−	6.0		−
$(C_6)n$	dextrine	##	6.2	LB	+
	inuline	##	5.8		−
	glycogen	+	5.6	YB	−

Y = yellow, B = brown, PY = pale yellow, LB = light brown, RB = reddish brown.

tion of the broth was recognized in about six carbon sources, i.e. glucose, fructose, mannitol, dextrine, gluconate and 2-ketogluconate. For the detection of oxidative products in the broth, the paper chromatography was adopted. A small amount of centrifuged fermentation liquor was freed of cations by the addition of ion exchange resin (Amberlite IR–120), and the liquor then dropped on a strip of Tōyō No. 50 filter paper. The papers were developed by the ascending method with a solvent mixture consisting of 4 volumes of *n*-Butanol, 1 volume of acetic acid and 5 volumes of water. After drying, it was sprayed with 1% solution of brom phenol blue or 1% solution of ferric chloride. From fructose and mannitol, the formation of three kinds of substances which gave positive ferric chloride reaction were recognized. One of them proved to be kojic acid, while the other two did not agree with any of the following rubiginol, rubiginic acid and comenic acid. The formation and chemical properties of these two substances will be published later in another paper. As it was proved that the dextrine contained a small amount of glucose, the formation of rubignol, rubiginic and comenic acid was confirmed to take place only in the case in which glucose, gluconate and 2-ketogluconate were used as the substrates.

Table II
Formation of γ-Pyrone Derivatives from Various Organic Acids by *Gluconoacetobacter liq.*

Cn	C-source	growth	pH	color*	$FeCl_3$
C_1	formate	−	6.0		−
C_2	acetate	−	6.0		−
C_3	pyruvate	−	6.0		−
〃	lactate	±	6.0		−
〃	malonate	+	6.0	PY	−
C_4	succinate	+	6.0	PY	−
〃	fumarate	−	6.0		−
〃	malate	+	5.8	Y	−
〃	oxalacetate	−	6.0		−
C_5	α-ketoglutarate	±	5.8		−
C_6	gluconate	##	4.8	RB	##
〃	2-ketogluconate	+	5.2	RB	##
〃	5-ketogluconate	+	5.8		−
〃	citrate	−	6.0		−
〃	*cis*-aconitate	−	6.0		−

* see Table I.

II. Oxidation of Glucose, Gluconate and 2-Ketogluconate by Intact Cell Suspensions. Manometric experiments were conducted with the Warburg manometer. The composition of the reaction mixture was as follows: substrate 5 μM, 1 ml of M/15 phosphate buffer (pH 6.0) and cell suspensions (intact cells of 2 mg dry-weight); total volume was 2.5 ml, temperature kept at 30°C and the gas phase was air. The results are shown in Fig. 1. Endogenous respiration was subtracted. Glucose, gluconate and 2-ketogluconate were oxidized rapidly with the evolution of carbon dioxide. But in the presence of 2×10^{-3} M 2,4-dinitrophenol (DNP), carbon dioxide evolution was not observed and 1.5, 1.0 and 0.5 moles of oxygen was consumed per mole of glucose, gluconate and 2-ketogluconate respectively as shown in Fig. 2.

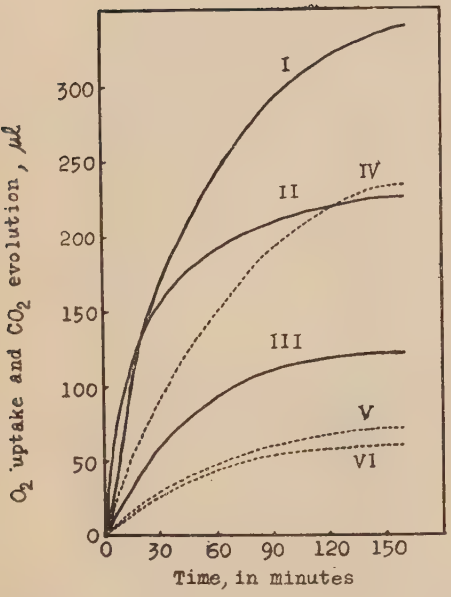


FIG. 1. Oxidation of Glucose, Gluconate and 2-Ketogluconate by Intact Cell Suspension of *Gluconoacetobacter liq.*

Each vessel contained, 0.5 ml of the cell suspension, 1 ml of phosphate buffer solution (M/15, pH 6.0) and 5μM of the substrate. Total volume, 2.5 ml. Temperature, 30°C.
oxygen uptake: I. glucose, II. gluconate, III. 2-ketogluconate
carbon dioxide evolution: IV. glucose, V. gluconate, VI. 2-ketogluconate

III. Oxidation by Cell-Free Preparation. *Gluconoacetobacter liq.* was grown in the culture liquid of 1% yeast extract-water containing 10% glucose and 2.5% calcium carbonate, for 40 hours at 30°C. After being harvested and washed, 1 g of the wet cells was suspended in M/15 phosphate buffer of pH 6.0 with 2 g of alumina, and the mixture treated with Potter-Elvehjem's homogenizer for a period of 20 minutes. After centrifuging the extract at 16,000 r.p.m. for 20 minutes, clear pale yellow supernatant was obtained, which was used for the experiment. Cell free extracts of *Gluconoacetobacter liq.* oxidized glucose, gluconate and 2-ketogluconate as shown in Fig. 3. Of the cell-free extracts 0.5 ml was used for the experiment, and other conditions were the same as described before. About 1.5, 1.0 and 0.5 moles of oxygen per mole of glucose, gluconate and 2-ketogluconate were consumed respectively. Carbon dioxide evolution was either negligible or not observed. Dried cell preparation was also prepared and it showed the same oxidative pattern to glucose, gluconate and 2-ketogluconate as

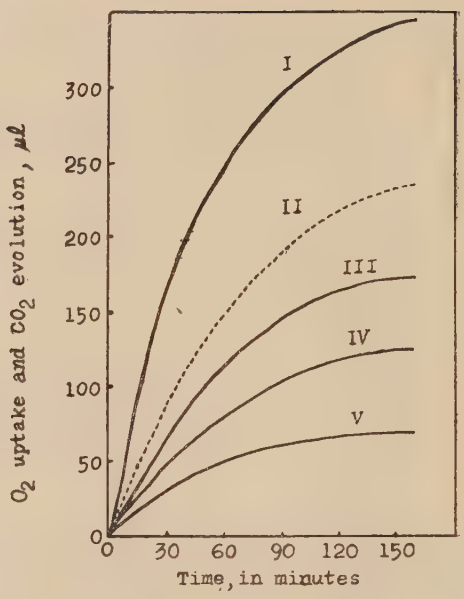


FIG. 2. Effect of 2,4-Dinitrophenol.

I. glucose (oxygen uptake),
II. glucose (carbon dioxide evolution),
III. glucose+DNP (oxygen uptake),
IV. gluconate+DNP (oxygen uptake),
V. 2-ketogluconate+DNP (oxygen uptake).

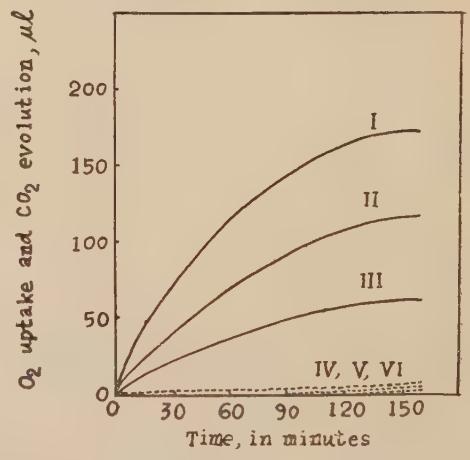


FIG. 3. Oxidation of Glucose, Gluconate and 2-Ketogluconate by Cell-free Preparation of *Gluconoacetobacter liq.*

oxygen uptake: I. glucose, II. gluconate, III. 2-ketogluconate.
carbon dioxide evolution: IV. glucose, V. gluconate, VI. 2-ketogluconate. Substrate, 5 μM.

cell-free extracts or intact cells in the presence of DNP.

IV. Effects of NaF and Phosphate. A quantity

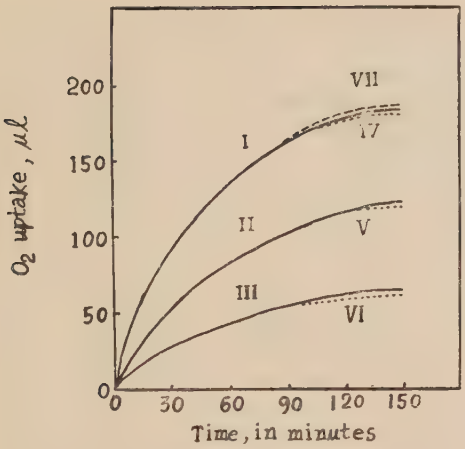


FIG. 4. Effects of NaF and Phosphate.
M/15 Phosphate buffer: I. glucose, II. gluconate, III. 2-ketogluconate, VII. glucose+NaF.
M/15 Phthalate buffer: IV. glucose, V. gluconate, VI. 2-ketogluconate.
Substrate, 5 μM. NaF, 5×10^{-2} M.

of 5 mg of dried cell was used. As shown in Fig. 4, NaF (5×10^{-2} M), which inhibits the transformation of phosphoglyceric acid to phosphopyruvic acid, did not influence either on the rate or on the total amount of oxygen uptake with glucose. The dissimilation of glucose, gluconate or 2-ketogluconate was essentially the same, both in its rate and extent of oxidation, whether phosphate or phthalate buffer (pH 6.0) were used. Glucose-1-phosphate, glucose-6-phosphate and 6-phosphogluconate were not dissimilated to an appreciable extent. These results suggest that glucose may not be phospholylated, prior to further dissimilation.

V. Oxidation Products from Glucose, Gluconate and 2-Ketogluconate. A 100 mg of the substrate was dissolved in 100 ml of M/15 phosphate buffer (pH 6.0), and was taken in a 500 ml shaking flask. Intact cells, 500 mg, were added to it and it was incubated at 30°C for 5 hours on a reciprocating shaker. After the cells were removed by centrifuging, broth was acidified with sulfuric acid and was extracted with ether. The ether soluble-fraction was examined by the application of paper chromatography. Organic acids and substances of positive ferric chloride reaction were detected. From glucose, gluconate and 2-ketogluconate, the same oxidation products, i.e. glycolic, tartronic, comenic, rubiginic

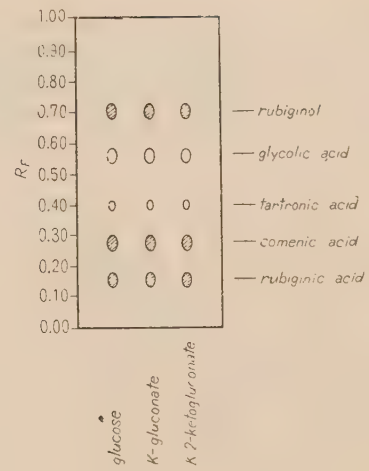


FIG. 5. Paperchromatogram of Oxidation Products from Glucose, Gluconate and 2-Ketogluconate by Intact Cell Suspension of *Gluconoacetobacter liq.*

acids and rubiginol were found, as shown in Fig. 5.

In the case of glucose, the formation of gluconate and 2-ketogluconate was identified by paper chromatography in the ether insoluble-fraction. 5-Ketogluconic acid was not detected in this experiment, while it was isolated and identified in the growing culture of this bacteria.

VI. Formation and Identification of 2,5-Diketogluconic Acid. Katznelson et al[7] reported that *Acetobacter melanogenum*, oxidized glucose via gluconate and 2-ketogluconate to 2,5-diketogluconate. We tried to detect 2,5-diketogluconate in the fermented liquid of glucose, gluconate and 2-ketogluconate by paper chromatography but could not find any spot corresponding to 2,5-diketogluconate from neither of them, and an unknown reducing acid, different from 2,5-diketogluconic acid, was detected and isolated as crude Ca-salt from the fermented liquid of glucose and gluconate. This acid reduces Fehling's solution and ammoniacal silver nitrate solution. By heating with orcin-hydrochloric acid, bluish green color occurs. The R_f value is 0.06–0.08 with a solvent system consisting of 4 volumes of *n*-butanol, 1 volume of acetic acid and 5 volumes of water. With aniline hydrogen oxalate as a developing agent, the unknown acid appeared as a pale-yellow spot, in contrary to the green-yellow spot of 2,5-diketogluconic acid and the ultraviolet absorption spectra were quite different

7) H. Katznelson, S.W. Tannenbaum and E.L. Tatum, *J. Biol. Chem.*, **204**, 43 (1953).

from that 2,5-diketogluconic acid. Further properties of this acid will be published later. 2,5-Diketogluconate was very unstable and decomposed very easily. So, we attempted to isolate it as follows: *Gluconoacetobacter liq.* was grown in shaking flasks at 30°C for 40 hours. The cells were centrifuged, washed twice with 0.85% NaCl solution and once with distilled water, then they were dried in desiccator for one night. By the use of a Warburg manometer, it was ascertained that 10 mg of dried cells consumed 0.5 moles of oxygen per mole of Ca-2-ketogluconate (5 μM) for about 20 minutes. A hundred mg of Ca-2-ketogluconate was dissolved in 50 ml distilled water and was taken in a 500 ml shaking flask. Dried cells, 150 mg, was added and it was incubated at 30°C for 30 minutes on a resiprocating shaker. The broth of five flasks was collected and centrifuged. Clear supernatant was lyophilized immediately. It was dissolved in a small quantities of water and precipitated by the addition of ethanol for three times. About 400 mg of pale yellow Ca-salt was obtained. The

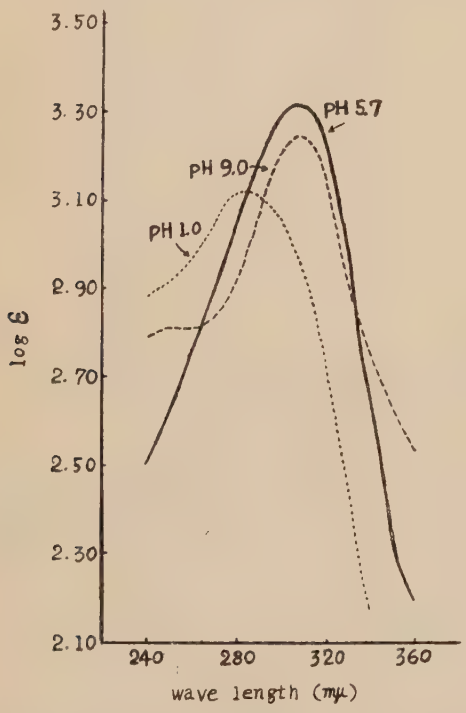


FIG. 6. Ultraviolet Absorption Spectra of Oxidation Product from Ca-2-ketogluconate by Dried Cell Preparation of *Gluconoacetobacter liq.*

absorption spectra of purified Ca-salt are shown in Fig. 6. λ_{max} at pH 5.7 was 305 mμ and λ_{max} at pH 1.0 was 280 mμ. By raising of the pH to 9.0 again, it showed λ_{max} of 305 mμ. These figures and shapes of absorption spectra quite agreed with those of Ca-2,5-diketogluconate which were reported by Katznelson et al.[5] Absorption of Ca-2-ketogluconate was negligible in the same concentration, also in the same range of wave length. The purity was tested by descending paper chromatography, using the solvent systems of water saturated isobutyric acid. Spots were detected by spraying aniline hydrogen oxalate. After 24 hours' flow this substance showed the R_F value of 0.54, while those of 2-ketogluconic and 5-ketogluconic acid were 0.77 and 0.87 respectively. This substances reduces Fehling's solution and ammoniacal silver nitrate in the cold. Pinoff's reaction, Seliwanoff's reaction, and sodium nitroprussid

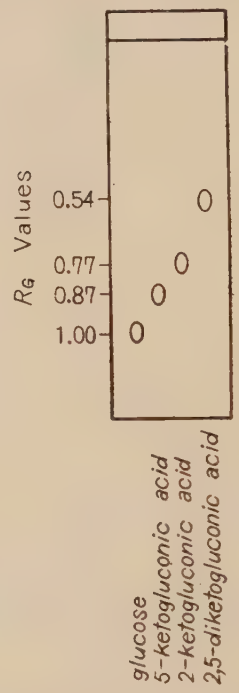


FIG. 7. Paperchromatogram of 2,5-Diketogluconic Acid Solvent: H_2O saturated isobutyric acid. Descending method.

reaction were all positive. It gave no ferric chloride reaction and the orcinol test was negative. From these data, this substance was identified as 2,5-diketogluconic acid.

VII. Decomposition of 2,5-Diketogluconic Acid by Cell Suspensions of *Gluconoacetobacter liq.* A hundred mg of the substrate was dissolved in 100 ml of M/15 phosphate buffer (pH 6.0) and dispensed in a 500 ml shaking flask. Then, 500 mg of intact cells was added to it and it was incubated at 30°C for 5 hours. Oxidation products were detected under the experimental condition, as described before. The three γ-pyrone derivatives corresponding to comenic acid, rubiginic acid and rubiginol besides glycolic and tartronic acids were detected by paper chromatography.

VIII. Formation of Rubiginol from Rubiginic Acid. Ten ml portions of medium composed of 0.8% yeast extract-water and 1% mannose were dispensed in two inverse-T-type shaking tubes, one of which contained 100 mg of rubiginic acid. Calcium carbonate 0.025 g, sterilized separately was added before inoculation. After inoculation with cells of *Gluconoacetobacter liq.*, the cultures were incubated for 10 days at 30°C on a resiprocating shaker. From the broth, containing rubiginic acid, rubiginol was detected by paper chromatography, but any substance that gives a positive ferric chloride reaction was not to be found in the control experiment.

DISCUSSION

With regard to the formation of three γ-pyrone derivatives, namely comenic acid, rubiginic acid and rubiginol, we have suggested in the previous paper[3], that they would be formed via the following pathway, i.e. glucose→gluconate→2-ketogluconate→2,5-diketogluconate.

As reported in this paper, we have examined the formation of the three γ-pyrone derivatives from thirty kinds of carbohydrates and fifty kinds of organic acids, which cover C_1, C_2, C_3, C_4, C_5, C_6, C_{12}, C_{13}, C_{18} compounds and three polysaccarides. In the case of kojic acid formation by *Aspergillus* species, some investigators[8,9,10] proposed a triose condensation theory. Dihydroxyacetone ($\rightleftarrows$ glyceradehyde) and a further hypothetical oxidation product, also C_3 compound 1,3-glyceroldialdehyde were presumed to condence in a manner analogous to Neuberg's aldol condensation of acetaldehyde by the enzyme carboligase in the biological genesis of acetylmethyl-carbinol. However, in our experiments, the formation of comenic acid, rubiginic acid or rubiginol was not observed from dihydroxyacetone or glyceraldehyde. From C_2 compound, such as ethanol, which was proposed to be an intermediate in the genesis of kojic acid[11,12] we could not find any of these three substances. Comenic acid, rubiginic acid and rubiginol were found to be formed only from glucose, gluconate and 2-ketogluconate. *Gluconoacetobacter liq.* oxidized glucose to gluconate and 2-ketogluconate, and it was also confirmed that 2-ketogluconate was oxidized further to 2,5-diketogluconate, which was isolated and identified. The formation of three γ-pyrone derivatives from 2,5-diketogluconate was also proved with intact cells. Therefore the three γ-pyrone derivatives were assumed to be formed via direct oxidative pathway as shown in the next page.

From analogy with the oxidation of glucose by notatin[13] or by an ox-liver[14] preparation, where gluconic acid lactone is the primary product, and the similar oxidation of glucose-6-phosphate to 6-phosphogluconolactone[15], it is tempting to postulate that the first stage may be the removal of two hydrogen atoms from carbon atom 1 of glucopyranose. Such oxidations, i.e. CHOH→CO, frequently occur in fungal and becterial metabolism as shown by the common occurence of ketogluconic acids, although usually only the 2- or 5-keto compounds are formed. Gluconic acid was oxidized to 2-ketogluconic and 2,5-diketogluconic acid. The formotion of 5-ketogluconic acid was

8) A. Corbellini and B. Gregorini, *Gazz. chim. ital.* **60**, 244 (1930).

9) F. Challenger L. Klein and T.K. Walker, *J. Chem. Soc.*, **1931**, 16

10) H. Katagiri and K. Kitahara, *Bull. Agr. Chem. Soc. Japan*, **5**, 38 (1929).

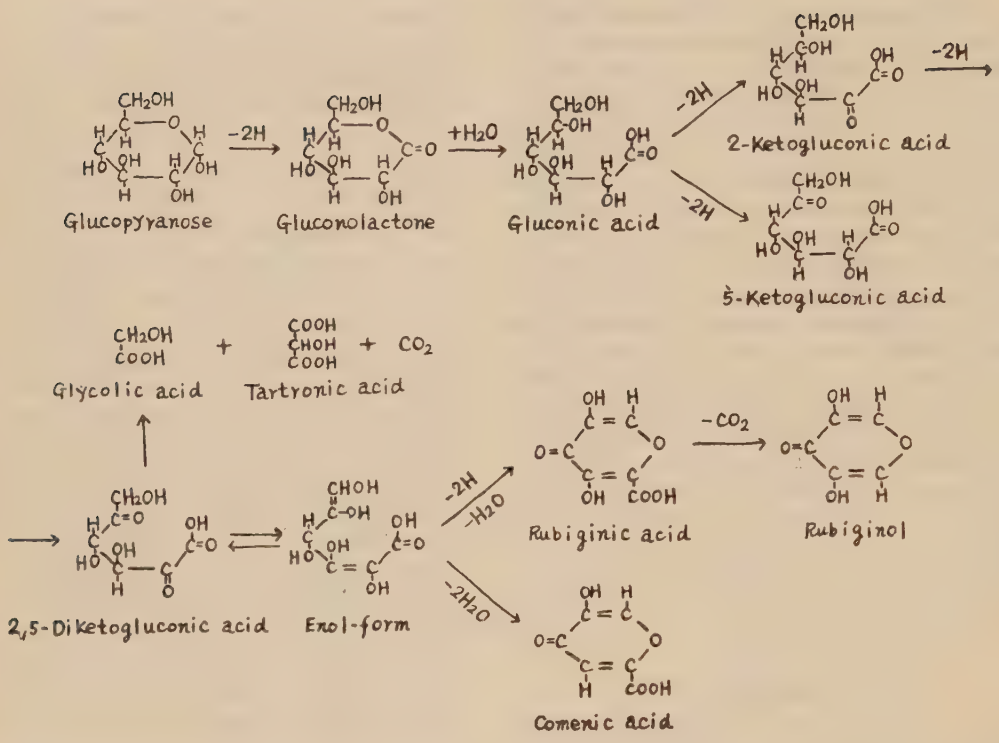
11) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **8**, 1265 (1932).

12) H.J. Birkinshaw and H. Raistrick, *Trans. Roy. Soc. London*, B **220**, 139 (1931).

13) R. Bentley and A. Neuberger, *Biochem. J.*, **45**, 584 (1949).

14) H.J. Strecker and S. Korkes, *Nature*, **168**, 913 (1951).

15) O. Cori and F. Lipmann, *J. Biol. Chem.*, **194**, 417 (1952).



also observed. The enol form of 2,5-diketogluconic acid was suggested by the reduction of 2,6-dichlorphenol-indophenol. Katznelson et al[7], also described the shift of absorption maximum towards lower wave-length upon acidification associated with enediol compounds such as ascorbic and dihydroxymaleic acid.

Concerning the formation of glycolic acid, a direct oxidation of acetic acid has been suggested by Bernhauer et al[16] in *Aspergillus niger*, and by Nord et al[17] in wood-destroying molds. But it can be readily supposed that glycolic acid is formed by the direct splitting of C_6 compound at the point of CO group, from the fact that glycolic acid was formed from 2- and 5-ketogluconic acids by shaking their aqueous solution on a resiprocating shaker non-enzymatically[17]. At least, it is sure to say that glycolic acid was formed via glucose→gluconate→2-ketogluconate→2,5-diketogluconate.

As it has been reported before[1,2], the unknown reducing organic acid was also found to be formed from glucose with a fairly high yield by this bacteria. We are now studying this acid, and it is supposed to be an intermediate product of the main pathway of glucose degradation by this bacteria.

16) K. Bernhauer and Z. Scheuer, *Biochem. Z.* **253**, 16 (1932).
17) F.F. Nord and J.C. Vitucci, *Advances in Enzymol.*, **8**, 253 (1948).

SUMMARY

1. Investigations were carried out on the formation of γ-pyrone derivatives, i.e. comenic acid, rubiginic acid and rubiginol from thirty kinds of carbohydrates, and fifteen kinds of organic acid. *Gluconoacetobacter liq.* was formed only from glucose, gluconate and 2-ketogluconate.

2. Intact cells oxidized glucose, gluconate and 2-ketogluconate with carbon dioxide evolution, but in the presence of DNP (2×

10^{-3} M) evolution of carbon dioxide was not observed, and 1.5, 1.0 and 0.5 moles of oxygen were consumed per mole of glucose, gluconate and 2-ketogluconate, respectively. Dried cells and cell-free extracts also showed the same oxidative pattern as intact cells in the presence of DNP.

3. Oxidation of glucose, gluconate and 2-ketogluconate by dried cells proceeded in phthalate buffer and was not affected by phosphate. Sodium fluoride (5×10^{-2} M) did not inhibit the oxidation of glucose.

4. Intact cells oxidized glucose, gluconate and 2-ketogluconate and formed the same oxidative products, i.e. the three γ-pyrone derivatives, and glycolic and tartronic acid.

5. As the oxidative product of Ca-2-ketogluconate by dried cells, Ca-2,5-diketogluconate was isolated and identified. *Gluconoacetobacter liq.*, formed the same oxidative products as described above from Ca-2,5-diketogluconate.

6. The chemical pathway of glucose degradation by *Gluconoacetobacter liq.* was discussed and the most presumable pathway involving the formation of γ-pyrone derivatives was given.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 38~42, 1957]

Studies on the Essential Oils of Tobacco Leaves

Part VII. Carbonyl Fraction (2).

By Isao Ōnishi and Masatoshi Nagasawa

Tobacco Research Department, Central Research Institute, Japan Monopoly Corporation

Received August 27, 1956

The presumption that some carbohydrates contained in the Virginia tobacco leaf converge to some furfural compounds in the stage of steam distillation was proved to be correct. The content of furfural compounds in the ether extractive-fraction was proved very much small in comparison with that in the essential oil. From the steam distillated essential oil, 5-hydroxymethylfurfural and acetone were newly isolated and identified. Besides these results, the presence of C_6-aldehyde was also estimated.

In the previous work[1], it was reported that the furfural compounds in the essential oil of tobacco leaf seemed not to be contained originally in the leaf itself, but to be derived from the carbohydrates in the leaf during the sampling process of the essential oil, employing steam distillation. In order to clarify this assumption, the present investigation was undertaken to isolate and identify the furfural compounds obtained by ether extraction from tobacco leaf without any steam distillating procedure, and the assumption was found right. Very small amoumts of furfural compounds were isolated from the ether extract.

In the previous paper, it was also reported that an unknown carbonyl compound was isolated besides acetaldehyde, isobutyraldehyde, benzaldehyde, furfural and 5-methylfurfural. However, this unknown high boiling point carbonyl compounds of Band No. 2 in Table II, described in Part II of this study was identified as 5-hydroxymethylfurfural by subsequent investigation.

In order to remove any objectionable compounds soluble in water, the ethereal solution containing the carbonyl compounds was washed with water in the process of isolating carbonyl compounds from other neutral ones in the previous work. Nevertheless, by this procedure, a part of some carbonyl compounds was found to be transferred into the water layer from the ethereal solution. Therefore, washing of the ethereal solution with water was omitted in the present investigation. Consequently, two carbonyl compounds were newly isolated and the former one was identified as acetone by its 2,4-dinitrophenylhydrazone (2,4-DNPHone).

EXPERIMENTAL

(I) Isolation of Carbonyl Compounds from Ether Extract

The same tobacco leaf (3.5 kg) as described in Part I[2] of this study was extracted with ether under reflux for twenty-four hours. The ethereal solution thus obtained, was treated by the same procedure as reported previously, but without washing the ethereal solution with water, after carbonyl compounds were separated with sulfuric acid from their sodium bisulfite compounds, so as to avoid any loss of the water soluble carbonyl compounds. From the low boiling point carbonyl fraction (I), 0.327 g of 2,4-DNPHone was obtained and similary, from the high boiling point one (II), 0.189 g of the same kind of derivative

1) I. Ōnishi and M. Nagasawa, This Bull., **19**, 143 (1955).

2) I. Ōnishi and K. Yamasaki, This Bull., **19**, 137 (1955).

Table I
Chromatographic Separation of 2,4-DNPHones Isolated From Low Boiling Point Carbonyl Fraction in the Ether Extracts

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
5	4.7	Unknown	Red
4	2.2	Furfural (*trans*-, *cis*-)	Orange-yellow
3	232.4	Acetaldehyde	Yellow
2	5.4	Benzaldehyde	Orange-yellow
1	2.9	Unknown	Yellow

Band numbers indicate the order of elution.
Adsorbent: Silicic acid+Celite (2:1 by Weight).
Column: 200×36 mm.
Developing solvent: 1-6% Ether in Petroleum benzine (b.p. 40-80°).

Table II
Chromatographic Separation of 2,4-DNPHones Isolated from High Boiling Point Carbonyl Fraction in the Ether Eetracts

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
6	101.6	Unknown	Red
5	10.0	Unknown	Red
4	6.8	Unknown	Red
3	3.1	Acetaldehyde	Yellow
2	2.6	Benzaldehyde	Orange-yellow
1	0.8	Unknown	Yellow

Foot-notes are the same as those of Table I.

was obtained.

(1) Chromatographic Separation of 2,4-DNPHones from Low Boiling Point Fraction (I): By the method described in Part II[1], 260 mg of the fraction (I) was separated by liquid chromatography. The results obtained are shown in Table I.

Band No. 1: A yellow crystal was obtained from Band No. 1. Although an infrared spectrum of this compound was essentially identical with that of the authentic isobutyraldehyde derivative, the melting point of this compound, 120°, did not agree with its authentic derivative. Further investigation on this compound is now in progress.

Identification of benzaldehyde, acetaldehyde and furfural: These compounds were identified as their 2,4-DNPHone crystals by their mixed melting points and infrared spectra with their authentic derivatives as described previously[1]. The details are summarized in Table V.

Band No. 5: This band could not be separated by any further developing.

(2) Chromatographic Separation of 2,4-DNPHones from High Boiling Point Fraction (II): By a similar method, as used in the former fraction, 130 mg of the fraction (II) was separated. The results obtained are shown in Table II.

Identification of benzaldehyde and acetaldehyde: These compounds were identified as their 2,4-DNPHone crystals by their mixed mlting points and infrared spectra with their authentic derivatives as described previously[1]. The details are summarized in Table V.

Band No. 1, 4 and 5: No crystalline derivatives could be isolated from these bands.

Band No. 6: This band could not be separated by any further developing.

(II) Isolation and Identification of 5-Hydroxymethylfurfural

An unknown carbonyl compound in Band No. 2 in Table II. described in Part II of this study was repeatedly collected by the same procedure employing a liquid chromatographic separation. Dark red crystals were obtained by repeated recrystallization from ethyl alcohol-ethyl acetate (1:1 by volume). The mixed melting point of this 2,4-DNPHone, m.p. 191°, with the authentic 5-hydroxymethylfurfural derivative, m.p. 194°, showed no depression and both infrared spectra agreed completely with each other. *Anal.* Found: N, 18.19. Calcd. for $C_{12}H_{10}O_6N_4$ (5-hydroxymethylfurfural 2,4-DNPHone): N, 18.30.

Table III

Chromatographic Separation of 2,4-DNPHones Isolated from Low Boiling Point Carbonyl Fraction Without Washing the Ether Solution with Water

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
7	1.0	Unknown	Red
6	9.6	Furfural (*trans*-, *cis*-)	Red
5	63.4	Acetaldehyde	Yellow
4	4.9	Acetone	Yellow
3	0.5	5-Hydroxymethylfurfural	Orange-yellow
	1.5	Benzaldehyde	Orange-yellow
2	16.8	Isobutyraldehyde	Yellow
1	6.4	Unknown	Yellow

Foot-notes are the same as those of Table I.

(III) Separation and Identification of Carbonyl Compounds Isolated from Carbonyl Fraction Without Washing the Ethereal Solution with Water

In the previous work the carbonyl compounds were separated with sulfuric acid from their sodium bisulfite derivatives and extracted with ether; the ethereal solution was washed twice with water (400 ml) and dried with sodium sulfate. As mentioned above, a part of the carbonyl compounds was transferred into the water layer. So that, washing of the ethereal solution with water was omitted in this work. The other procedure was undertaken in the same way as described previously. From the low boiling point carbonyl fraction (I), 1.10 g of 2,4-DNPHone was obtained, and similary, from the high boiling point one (II), 6.10 g of the same kind of derivative from 100 kg of the original tobacco leaf was obtained.

(1) Chromatographic Separation of 2,4-DNPHones from Low Boiling Point Fraction (I): By the method described in Part II[1], 110 mg of the fraction (I) was separated by liquid chromatography. The results obtained are shown in Table III.

Estimation of C_6-aldehyde: A yellow crystal, m.p. 127–8°, was obtained from Band No. 1. No such carbonyl 2,4-DNPHones, having a melting point as that, was found in the literature. Infrared spectrum

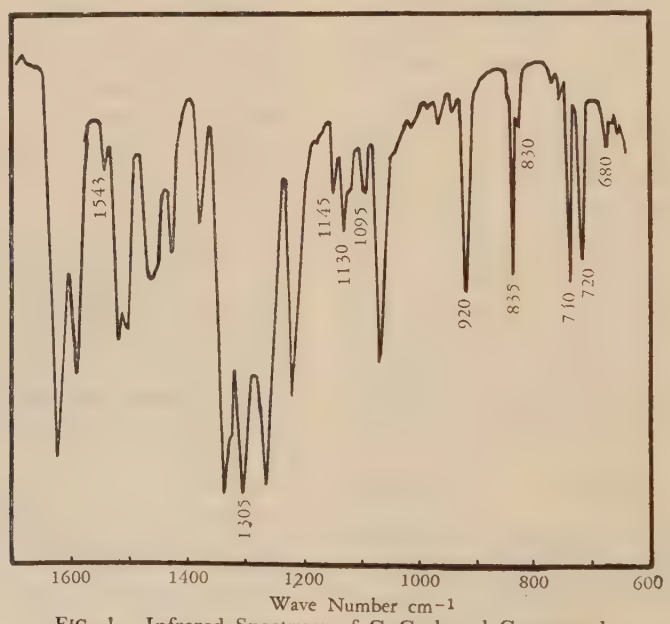


Fig. 1. Infrered Spectrum of C_6-Carbonyl Compound.

TABLE IV

CHROMATOGRAPHIC SEPARATION OF 2,4-DNPHONES ISOLATED FROM HIGH BOILING POINT CARBONYL FRACTION WITHOUT WASHING THE ETHER SOLUTION WITH WATER

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
8, 7	4.6	Unknown	Red
6, 5	1.1	Unknown	Red
4	13.8	Furfural (*trans*-)	Red
3	7.8	5-Hydroxymethylfurfural	Dark red
2	8.2	Furfural (*cis*-)	Yellow
1	6.0	5-Methylfurfural	Orange-yellow
	3.1	Benzaldehyde	Orange-yellow

Foot-notes are the same as those of Table I.

of this derivative is illustrated in Fig. 1. *Anal.* Found: N, 19.79. Calcd. for $C_{12}H_{16}O_4N_4$ (as C_6-aldehyde derivative): N, 20.00. Further investigation on the compound is now in progress.

Identification of benzaldehyde and 5-hydroxymethylfurfural: As employed in Band No. 2 in Table I of Part II, Band No. 3 was separated into two bands by a subsequent chromatographic procedure. An orange-yellow crystal, m.p. 242°, was obtained from the bottom band. The mixed melting point with the authentic derivative of benzaldehyde, m.p. 243°, showed no depression and both infrared spectra were also identical with each other. From the upper band, an orange-yellow crystal, m.p. 191°, was obtained. The mixed melting point with the authentic 5-hydroxymethylfurfural, m.p. 194°, showed no depression and both infrared spectra were also identical with each other.

Identification of acetone: A yellow crystal, m.p. 120°, was obtained from Band No. 4 by four successive recrystallizations from 60% ethyl alcohol. The mixed melting point with the authentic derivative of acetone, m.p. 127°, showed no depression and both infrared spectra were also identical with each other.

Identification of isobutyraldehyde, acetaldehyde and furfural: These compounds were identified as their 2,4-DNPHone crystals by their mixed melting points and infrared spectra with their authentic derivatives as described previously[1]. The details are summarized in Table V.

Band No. 7: This band could not be separated by any further developing.

(2) Chromatographic Separatoin of 2,4-DNPHones from High Boiling Point Fraction (II): By the method described in Part II[1], 40 mg of the fraction (II) was separated by liquid chromatography. The results obtained are shown in Table IV.

Identification of benzaldehyde and 5-methylfurfural: By the method used in Band No. 2 in Table I of Part II., Band No. 1 was separated into two bands by

TABLE V

IDENTIFICATING RESULTS OF THE CARBONYL COMPOUNDS IN TABLE I–IV

Table	Band No.	Isolated Crystal of 2,4-DNPHone	m.p. of Isolated Specimen	m.p. of Isolated Specimen	Mixed m.p. Depression	Infrared Spectrum with Authentic Specimen
I	2	Benzaldehyde	240	243	None	Identical
	3	Acetaldehyde	159	164	None	Identical
	4	Furfural (*trans*-, *cis*-)	220	223	None	Identical
II	2	Benzaldehyde	239	243	None	Identical
	3	Acetaldehyde	158	164	None	Identical
III	2	Isobutyraldehyde	182	186	None	Identical
	5	Acetaldehyde	159	164	None	Identical
	6	Furfural (*trans*-, *cis*-)	219	223	None	Identical
IV	2	Furfural (*cis*-)	204	206	None	Identical
	3	5-Hydroxymethylfurfural	191	194	None	Identical
	4	Furfural (trans-)	229	231	None	Identical

subsequent chromatographic procedure. An orange-yellow crystal, m.p. 242°, was obtained from the bottom band. The mixed melting point with the authentic derivative of benzaldehyde, m.p. 243°, showed no depression and both infrared spectra were also identical with each other. From the upper band, an orange-yellow crystal, m.p. 221°, was obtained. The mixed melting point with the authentic derivative of 5-methylfurfural, m.p. 223°, showed no depression and both infrared spectra were also identical with each other.

Identification of 5-hydroxymethylfurfural and furfural: These compounds were identified as their 2,4-DNPHone crystals by the same procedure as mentioned above. The details are summarized in Table V.

Bands No. 5, 6, 7 and 8: No crystalline derivatives could be isolated from these bands.

RESULTS AND DISCUSSION

The contents of carbonyl compounds in the ether extracts are shown in Table VI, and those of carbonyl compounds in the essential oil without washing the ethereal solution with water are shown in Table VIII.

In the ether extractive fractions the low boiling point fraction of the carbonyl compound contains a large amount of acetaldehyde as a predominant constituent and the quantity of furfural is very small. However,

TABLE VI
CONTENTS OF CARBONYL COMPOUNDS FOUND IN THE ETHER EXTRACTS OF VIRGINIA TOBACCO LEAF

Constituents	%	mg/kg of leaf
Acetaldehyde	49.3	16.7
Benzaldehyde	3.3	1.1
Furfural	0.9	0.3
Unknown	46.5	15.7
Total	100.0	33.8

TABLE VII
CONTENTS OF CARBONYL COMPOUNDS FOUND IN THE CARBONYL FRACTION OF THE ESSENTIAL OIL OF VIRGINIA TOBACCO LEAF

Constituents	%	mg/kg of leaf
Furfural	50.4	11.8
5-Methylfurfural	14.6	3.4
Benzaldehyde	7.6	1.8
5-Hydroxymethylfurfural	7.5	1.8
Acetaldehyde	5.3	1.3
Isobutyraldehyde	2.0	0.5
C_6-aldehyde	1.0	0.3
Acetone	0.5	0.1
Unknown	11.1	2.6
Total	100.0	23.6

the high boiling point fraction of the above mentioned extracts consists mostly of a mixture of some syrupy substances, and the presence of furfural is not ascertained.

In view of these facts, it seemed to be the most authentic statement, as presumed in the previous work, that a greater part of those furfural compounds was derived from the carbohydrates by the steam distillation. Moreover, the isolation of 5-hydroxymethylfurfural suggested some support to this assumption.

The syrupy substances, contained in the high boiling point fraction, were assumed to be the intermediate decomposed complex compounds, derived from carbohydrates and changing to the furfural compounds by steam distillation, while, on the other hand, a small amount of furfural compound was found in the ether extractive fraction. Consequently, it was assumed that these changes took place to some extent during the stage of storage of fermentation of the tobacco leaves.

There was noticeable some difference between the total amount and ratios of each quantity of the two carbonyl fractions, namely — the one isolated after washing the ether solution with water as the former method described in the previous paper, and the other isolated directly from it as the latter one in this paper. Comparing these two procedures, it seems that the latter method is more reasonable to discuss the contents of the carbonyl compounds in the essential oil of the tobacco leaves.

Acknowledgement. We wish to express our cordial thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo for his wise direction and kind guidance, also to Director T. Nakashima, of the Central Research Institute, Japan Monopoly Corporation for his strong support given. We are indebted to Dr. M. Matsui, Department of Agricultural Chemistry, University of Tokyo, for the microanalysis and Miss K. Yamamoto and Mr. K. Saito for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. T. Yabuta for his sound advice.

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Studies on the Essential Oils of Tobacco Leaves

Part VIII. Carbonyl Fraction (3)

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The constituents and contents of the carbonyl compounds, contained in the essential oil of Virginia tobacco leaves, were compared before and after redrying and aging. In the course of the redrying and aging stage, furfural, isobutyraldehyde and the estimated C_6-aldehyde decreased, while, on the contrary, 5-hydroxymethylfurfural and 5-methylfurfural increased. Acetone is the only constituent that has been found after redrying and aging.

In the previous work[1], it was reported that furfural, 5-methylfurfural, benzaldehyde, 5-hydroxymethylfurfural, acetaldehyde, isobutyraldehyde and acetone were isolated and identified, and the presence of C_6-aldehyde was also estimated in the essential oil of redried and fermented Virginia tobacco leaf. In order to compare the changes of these carbonyl compounds in the course of redrying and aging stages, the present investigation was undertaken to isolate and identify these carbonyl compounds in the essential oil of tobacco leaf before redrying. The same kind of tobacco leaf of the 1955 crop, as that described in Part I[2] of this study, was employed as the original sample. Bacause of the difference of the harvested year, in the case of Part I of the 1952 crop, the same sample in this study was packed in hogsheads for the purpose of investigation of comparing the strict differences before and after redrying and aging.

In the essential oil, obtained from this Virginia tobacco leaf before redrying, furfural, 5-methylfurfural, benzaldehyde, 5-hydroxymethylfurfural, acetaldehyde, isobutyraldehyde and C_6-aldehyde were also isolated and identified as well as in the case of after redrying and aging, as it has been described in Part VII of this study. However, only acetone was not isolated.

1) I. Ōnishi and M. Nagasawa, This Bull., **20**, (1956).
2) I. Ōnishi and K. Yamasaki, This Bull., **19**, 137 (1955).

EXPERIMENTAL

(I) Separation and Identification of the Carbonyl Compounds Before Redrying

The Virginia tobacco leaves before redrying, 134 kg, were treated by the method similar to that described in Part I. The 2,4-dinitrophenylhydrazones (2,4-DNPHones) of the carbonyl compound were prepared and fractionated by the method similar to that described in Part VII of this study. From the low boiling point carbonyl fraction (I), 1.80 g of 2,4-DNPHone was obtained and similary, from the high boiling point one (II), 8.67 g of the same kind of derivative was obtained.

(1) Chromatographic Separation of 2,4-DNPHone from Low Boiling Point Fraction (I): By the method described in Part II, 130 mg of fraction (I) was separated by liquid chromatography. The results obtained are shown in Table I.

Band No. 1: A yellow crystal was obtained from Band No. 1. The infrared spectrum and mixed melting point indicated that this compound was identical with that of Band No. 1 in Table III of the previous Part VII, which was estimated as C_5-aldehyde.

Band No. 3: This band was separated into three bands by subsequent chromatographic procedure as

TABLE I

CHROMATOGRAPHIC SEPARATION OF 2,4-DNPHONES ISOLATED FROM LOW BOILING POINT CARBONYL FRACTION IN ESSENTIAL OIL OF TOBACCO LEAF BEFORE REDRYING

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
5	4.6	Furfural (*trans*-, *cis*-)	Red
4	63.4	Acetaldehyde	Yellow
3	0.2	5-Hydroxymethylfurfural	Dark red
	1.0	5-Methylfurfural	Orange-yellow
	1.4	Benzaldehyde	Orange-yellow
2	40.5	Isobutyraldehyde	Yellow
1	16.3	Unknown	Yellow

Band numbers indicate order of elution.
Adsorbent: Silicic acid + Celite (2 : 1 by weight).
Column: 200×36 mm.
Developing solvent: 1-6% Ether in Petroleum benzine (b.p. 40–80°).

TABLE II

CHROMATOGRAPHIC SEPARATION OF 2,4-DNPHONES ISOLATED FROM HIGH BOILING POINT CARBONYL FRACTION IN ESSENTIAL OIL OF TOBACCO LEAF BEFORE REDRYING

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
5	4.9	Unknown	Red
4	84.2	Furfural (*trans*-)	Red
3	3.5	5-Hydroxymethylfurfural	Dark red
2	10.0	Furfural (*cis*-)	Yellow
1	7.7	5-Methylfurfural	Orange-yellow
	6.6	Benzaldehyde	Orange-yellow

Foot-notes are the same as those of Table I.

TABLE III

IDENTIFICATING RESULTS OF THE CARBONYL COMPOUNDS IN TABLES I AND II

Table	Band No.	Isolated Crystal of 2,4-DNPHone	m.p. of Isolated Specimen	m.p. of Authentic Specimen	Mixed m.p. Depression	Infrared Spectrum with Authentic Specimen
I	2	Isobutyraldehyde	180	186	None	Identical
	3	Benzaldehyde	241	243	None	Identical
		5-Methylfurfural	221	223	None	Identical
		5-Hydroxymethylfurfural	190	194	None	Identical
	4	Acetaldehyde	159	164	None	Identical
	5	Furfural (*trans*-, *cis*-)	220	223	None	Identical
II	1	Benzaldehyde	241	243	None	Identical
		5-Methylfurfural	220	223	None	Identical
	2	Furfural (*cis*-)	206	206	None	Identical
	3	5-Hydroxymethylfurfural	191	194	None	Identical
	4	Furfural (*trans*-)	229	231	None	Identical

employed in Band No. 2 in Table I of Part II.

All isolated 2,4-DNPHone crystals were identified by their mixed melting points and infrared spectra with their authentic derivatives, as described previously. The details are summarized in Table III.

(2) Chromatographic Separation of 2,4-DNPHones from High Boiling Point Fraction (II): By the method described in Part II, 120 mg of the fraction (II) was separated by liquid chromatography. The results obtained are shown in Table II.

Band No. 1: After subsequent application of chromatographic procedure, two bands were separated by just the same method as employed in Band No. 1 in Table IV of the previous Part VII.

TABLE IV
COMPARISON OF CONTENTS OF CARBONYL COMPOUNDS IN ESSENTIAL OIL OF VIRGINIA TOBACCO LEAF BEFORE REDRYING AND AFTER REDRYING AND ONE YEAR-AGING

Constituent	Before Redrying		After Redrying and One Year-Aging	
	%	mg/kg of leaf	%	mg/kg of leaf
Furfural	70.0	17.8	50.4	11.8
5-Methylfurfural	6.3	1.6	14.6	3.4
Benzaldehyde	5.4	1.4	7.6	1.8
Acetaldehyde	5.0	1.3	5.3	1.3
Isobutyraldehyde	4.7	1.2	2.0	0.5
5-Hydroxymethylfurfural	3.1	0.8	7.5	1.8
C_6-aldehyde	2.4	0.6	1.0	0.3
Acetone	none	none	0.5	0.1
Unknown	3.1	0.8	11.1	2.6
Total	100.0	25.5	100.0	23.6

Band No. 5: This band could not be separated by any further developing.

All the isolated 2,4-DNPHone crystals were identified by their mixed melting points and infrared spectra with their authentic derivatives as described previously. The details are summarized in Table III.

RESULTS AND DISCUSSION

A comparison of the quantities between before and after redrying and aging of the carbonyl compounds contained in the essential oil of Virginia tobacco leaf are shown in Table IV.

It was found that the total carbonyl compounds have decreased during the stage of redrying and aging. Furfural showed the most remarkable decrease, corresponding to just two-thirds of the content prior to redrying. Whereas, on the other hand, 5-methylfurfural and 5-hydroxymethylfurfural have increased two times, respectively. However, because the content of furfural is far too high as compared with that of the other constituents, the total amounts of furfural compounds have decreased. Besides, isobutyraldehyde and C_6-aldehyde have decreased by half. Acetaldehyde and benzaldehyde have not changed. Acetone was not to be found prior to redrying, but it was found after redrying and aging.

The redried Virginia tobacco leaf, employed in this investigation, was packed into the hogshead with a moisture content of eleven percent and aged for one year. According to recent studies in our laboratory conducted on the redrying and aging of Virginia tobacco leaf under such conditions as these, it has been shown that water soluble-sugars have decreased, while, on the contrary, water insoluble-sugars have increased, as illustrated in Table V.

Although the individual constituents of the water soluble- and water insoluble-sugars are not clarified at the present, some presumption may be possible that furfural compounds

TABLE V
CHANGES OF SUGAR CONTENTS DURING REDRYING AND AGING OF VIRGINIA TOBACCO LEAF

Constituent	Before Redrying %	After Redrying %	After Aging for One Year %
Water soluble-sugar	20.97	20.67	16.71
Water insoluble-sugar	1.80	3.04	6.16
Total sugar	22.77	23.71	22.87
	Change of ratios		
Drymatter of leaf	100.00	99.87	98.08
Water soluble-sugar	100.00	98.47	78.16
Water insoluble-sugar	100.00	168.89	335.56
Total sugar	100.00	104.00	97.23

were derived from some carbohydrates, because that the decrease of both constituents have come to show the same tendency in tobacco leaf, as well as in other plants.

Acknowledgement. We wish to express our cordial thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support given. We are also indebted to Miss K. Yamamoto and Mr. K. Saito for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. T. Yabuta for his sound advice.

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Studies on Microbial Biosynthesis of Pantothenic Acid

Part V. Pantothenate Synthesis from Pantoate and β-Alanine by *Escherichia coli* Extracts

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Received September 5, 1956

Pantothenate synthesis from pantoate and β-alanine by *Escherichia coli* extracts was studied as a model synthesis of the peptide bond. It was found as the evidence for occurence of pantoyl-carboxyl activation that a significant quantity of pantoic acid hydroxamate was formed in the reaction mixture containing pantoate and ATP. Formation of pantothenate and development of hydroxamate color from activated pantoate were increased under the anaerobical conditions. Inhibition by -SH reagents and reversal of inhibition by thiol compounds are indicative of the evidence for involvement of -SH enzyme in pantothenate synthesis and pantoate activation. In view of these results, pantoate activation is formulated as shown in the following two-step reaction:

(1) $HS\text{–}E+AMP\sim PP \rightleftarrows PP\sim S\cdot E+AMP$

(2) $PP\sim S\cdot E+C_5H_{11}O_2\cdot COO^- \rightleftarrows C_5H_{11}O_2\cdot CO\sim S\cdot E+PP.$

It has been noted that pantothenate synthesis from pantoate and β-alanine represents a model peptide synthesis. Regarding this interesting reaction, Maas and Novelli[1] have shown that the overall reaction is: pantoate + β-alanine + ATP → pantothenate + AMP + PP. Maas[2,3] reported that formation of this peptidic linkage seems not to involve coenzyme A, and presented the following sequence as the mechanism of this reaction on the basis of the successful use of isotope exchange.

(1) $E+ATP \rightleftarrows E\text{-}PP+AMP$

(2) $E\text{-}PP+\text{pantoate} \rightleftarrows E\text{-pantoate}+PP$

(3) $E\text{-pantoate}+\beta\text{-alanine} \rightleftarrows \text{pantothenate}+E$

It has been presumed that pantoyl-carboxyl activation initiates peptide bond formation, because pyrophosphate exchange was promoted by pantoate, but not by β-alanine. In the hope of trapping the activated carboxyl group of the intermediary compound containing the enzyme, hydroxamate formation was employed. The data indicated in this paper suggest that pantoyl-carboxyl activation occurs at the expense of the pyrophosphate bond of ATP and pantothenate synthesis takes place anaerobically in the presence of ATP.*

Abbreviations: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; PP, pyrophosphate; P, orthophosphate; CoA, coenzyme A; HSCoA, sulfhydryl-coenzyme A; E, enzyme; HS-E, -SH enzyme.

* The data indicated in the previous communication (This Bulletin, **20**, 99 (1956)) were corrected as shown in this paper, in concern of the formation of pantoyl hydroxamate.

1) W.K. Maas and G.D. Novelli, *Arch. Biochem. and Biophys.*, **43**, 236 (1953).

2) W.K. Mass: *J. Biol. Chem.*, **198**, 23 (1952).

3) W.K. Mass: *Federation Proc.*, **13**, 256 (1954)

MATERIALS AND METHODS

Enzyme Source and Reagents. The source of the enzyme was the wild type strain of *Escherichia coli* K 12 which was supplied from the Institute of Applied Microbiology, University of Tokyo. The bacteria were grown on the broth agar at 37° for 20 hours. Potassium pantoate was prepared by dissolving optically active lactone in KOH in a 1:4 molar ratio, and after heating at 100° for 15 minutes, the pH was brought to 8.5 with dil. HCl as previously described by Maas[2]. As pantolactone forms hydroxamate in alkaline solution, complete cleavage of the lactone

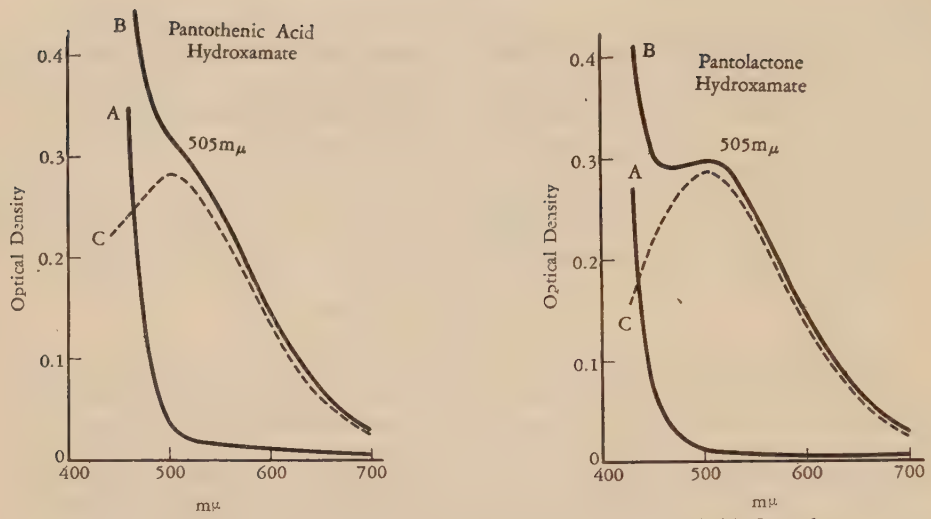

FIG. 1. Absorption of Visible Light by Ferric-Hydroxamic Acid Complex.

Hydroxamic acid were formed from: 3.0μM of (+)-pantothenic acid and 1.5μM of (−)-pantolactone, respectively; A, reagent blank; B, total extinction; C, absorption by ferric-hydroxamic acid complex; Final volume, 5.0ml; pH, 7.

ring is needed to avoid interference of the lactone in determining the hydroxamate from activated pantoate. Potassium adenosinetriphosphate was prepared by dissolving barium salt with the addition of 3 N H_2SO_4 in the cold, and neutralizing the supernatant with 3 N KOH. Barium salt of ATP, β-alanine and calcium salt of pantothenate were chromatographically pure.

Extraction of Enzyme. The enzyme was extracted from acetone-dried bacteria which was approximately obtained according to the method of Maas. For extraction, the acetone-dried powder was suspended in 20–30 times its weight of 0.025 M Tris buffer (pH 7.0) containing 10 μM KCl and 2.5 μM $MgCl_2$ per ml, at 5° for about one hour. After centrifuging by the angle head centrifuge at 6000 r.p.m., the extracts were dialyzed against distilled water at 5° for 24 hours.

Determination of Inorganic Phosphorus and Acid-labile Organic Phosphate. At the end of incubation the enzyme action was stopped by treatment with 6% trichloroacetic acid at 0° and then the pH of the solution was brought to around 8.8 by the addition of dil. NaOH. Inorganic phosphorus was precipitated by employing a $CaCl_2$ reagent[4] and determined according to the method of King[5] after hydrolysis of the precipitate was carried out for 15 minutes at 100° in 1 N HCl. Acid-labile organic phosphate was similarly determined on the supernatant hydrolysed for 15 minutes in 1 N HCl which was separated from the precipitate mentioned above. The optical density at 680 mμ was given as 0.116 per μg of phosphorus in this study.

Estimation of Pantothenate. Pantothenate estimation was carried out by the method of Bergmann[6] as the hydroxamate and by that of the microbiological assay. Hydroxamate formation from pantothenate was performed in the strong alkaline solution after heating, and under these conditions it was found that active pantoate cannot develop the hydroxamate color because of its lability to heat and alkalinity as reported later. Absorption of the visible light by the ferric-hydroxamate of pantothenic acid complex indicated the maximum at 505 mμ (Fig. 1). The color varies along with the pH of the solution, the optimum being 1.2 (Fig. 2), and it is somewhat unstable. In this case, it is preferable to add ferric chloride reagent directly to the reaction mixture as pointed out by Hoagland[7] in the case of determination of the amino acid hydroxamate. The procedure for pantothenate estimation by means of hydroxamate formation was as follows: 1 ml of the enzymatic

4) W.W.Umbreit, Manometric Techniques and Tissue Metabolism, 190 (1951).

5) E.J. King, *Biochem. J.*, **26**, 292 (1932).

6) F. Bergmann, *Anal. Chem.*, **24**, 1367 (1952).

7) M.B. Hoagland, *Biochem. et Biopys, Acta*, **16**, 288 (1955).

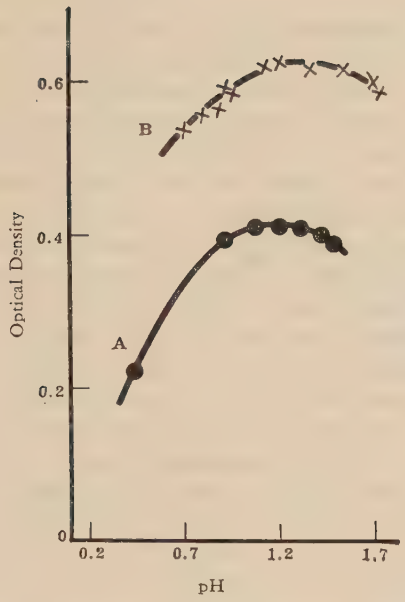

FIG. 2. Influence of Acidity on Hydroxamate Color

The color was developed in a solution of 5ml containing the hydroxamic acid form 3μM (+)-pantothenic acid (A) or 2μM (−)-pantolactone (B), and measured at 505mμ.

reaction mixture was heated at 100° for 2 minutes and added with 2 ml of the alkaline hydroxylamine reagent, which was prepared by mixing equal volumes of 2 N hydroxylamine sulfate or hydrochloride and 3.5 N NaOH. The mixture was held at 25° for 5 hours. Two ml of the ferric chloride reagent was added to the cooled incubation mixture. The ferric chloride reagent for determination of pantothenate was prepared by mixing equal volumes of ca. 4 N HCl, 40% trichloroacetic acid, water and 1.48 M ferric chloride in a 0.2 N HCl-KCl buffer (pH 1.2). Normality of the HCl mentioned above was adjusted prior to use to that the resultant pH might be brought to around 1.2. The final mixture was centrifuged at 3000 r.p.m. and the optical density at 505 mμ of the clear solution was strictly read 5 minutes after the addition of the ferric chloride reagent. The extinction values were corrected for the blank absorption.

The microbiological assay of pantothenate was carried out by using *Lactobacillus arabinosus* ATCC 8014, which required pantothenate even in the presence of pantoate and β-alanine. The reaction mixture inactivated by heating was diluted in 10,000–80,000 times with water, and 1 ml of the sample was added to 1 ml of the basal medium. The mixture was cultivated by the ordinary procedure for microbiological assay and pantothenate was determined by turbidimetry.

Formation and Determination of Pantolactone Hydroxamic Acid. Absorption maximum of ferric-hydroxamate of (−)-pantolactone complex was at 505 mμ as well as in the case of pantothenate (Fig. 1). The color density varies with the pH, the optimum being 1.2 (Fig. 2). The relationship between the color density at 505 mμ and the reaction time is shown in Fig. 3. Formation of hydroxamic acid from pantolactone is considerably influenced by the pH and the temperature. But the addition of KCl greatly eliminates influence of the temperature, this probably being due to the effect on the cleavage of the lactone ring. Two methods for estimation of pantolactone hydroxamic acid in the Tris buffer pH 9.0 were carried out. Procedure I — One ml of the sample was mixed with 1 ml of a 2 N hydroxylamine reagent at pH 9.4 containing 6% KCl which was prepared by the addition of 3.5 N NaOH to 4 N hydroxylamine hydrochloride solution. After the mixture was kept at −5° for 10 hours, 3 ml of the ferric chloride reagent was prepared by mixing 6 ml of 1 N HCl, 10 ml of 40% trichloroacetic acid, 34 ml of water and 10 ml of 1.48 M ferric chloride in a 0.2 N HCl-KCl buffer solution (pH 1.2). Acidity of the reagent was adjusted in advance so as to bring the resultant pH around to 1.2. The optical density of the

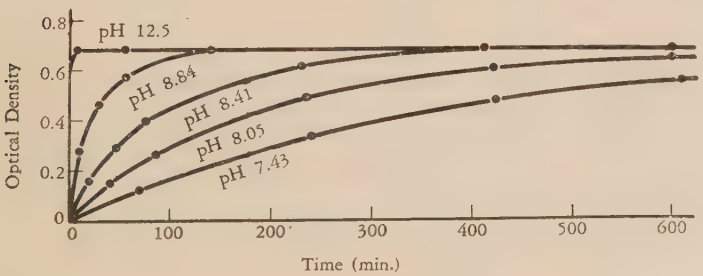

FIG. 3. Relationship Between Reaction Time and Hydroxamate Color Formed from (−)-Pantalactone

Hydroxamate was formed from 2.2μM of pantolactone in a solution of 4ml at room temperature and the color was measured at 505 mμ. The pH values indicated are those of the reaction mixture.

solution obtained was read at 505 mμ after 5–10 minutes. Procedure II — After incubation with the hydroxylamine reagent the sample was added successively with 1 ml of water and each 0.5 ml of 3 N HCl, 40% trichloroacetic acid, 2.0–2.5 N NaOH and 1.48 M ferric chloride in the order just mentioned. The color density was read similarly to Procedure I. It was interesting to observe that in this case the optical densities obtained by the two methods were identical, but the hydroxamate color which is presumed to be responsible for activated pantoate gave different values as described below.

Estimation of Pantoyl Hydroxamate in the Enzymatic Reaction Mixture. Appearance of pantoyl hodroxamate in the enzymatic reaction mixture was determined by the method according to procedure II of pantolactone hydroxamate estimation because of the decrease in the hydroxamate color obtained by procedure I. The procedure was as follows: At the end of the enzymatic reaction, 1 ml of the sample was rapidly cooled by the freezing mixture and added with 2 ml of the cold 1 N hydroxylamine reagent pH 9.4 containing Ba^{++} (added with 2% $BaCl_2$ and filtered). After it was kept at $-5°$ for 10 hours, a mixture of 0.5 ml of 3 N HCl and 40% trichloroacetic acid was added to the sample solution. The solution was then centrifuged at 4000 r.p.m. The clear solution obtained was decanted, added with 0.5 ml of 1.2 N NaOH and then 0.5 ml of 1.48 M ferric chloride in 0.2 M HCl-KCl buffer solution (pH 1.2). The color density was read at 505 mμ after 5 minutes and compared to a zero-time control, using hydroxamate of (−)-pantolactone as a relative standard. With the enzymatic reaction mixture a thick scum at the top of the tube may appear after centrifuging so, it is necessary to treat the reaction mixture in the cold and decant it very carefully to obtain a clear solution. By this procedure pantothenate cannot produce the hydroxamate color because of the lower pH of the incubation mixture added with the hydroxylamine reagent.

Apparatus. The spectrophotometric measurements were made in the Beckmann D.U. spectrophotometer with 1 cm cells. Turbidimeter was carried out by using the Coleman Nepho-colorimeter model 9. The pH measurements were obtained with the Beckmann model G pH meter standardized with the buffers having the pH of 4 and 7.

RESULTS

Evidence for Occurence of Pantoate Activation. The main evidence for activation of the pantoyl-carboxyl group was obtained by an experiment of hydroxamate formation. In order to establish the method for the determination of activated, hydroxamate formation and its determination of (−)-pantolactone were studied, for the hydroxamate of pantoate with the carboxyl group must to identical with that of (−)-pantolactone. It was illustrated that the maximum color of the ferric-hydroxamate complex was achieved at 505 mμ and pH 1.2. The formation of pantolactone hydroxamate is promoted as an increase in alkalinity of the incubation mixture, but this observation seems not to be available for hydroxamate formation from activated pantoate. With hydroxamate formation from pantolactone the cleavage of the lactone ring is needed. As the complex containing activated pantoate might be sensitive to the pH of the solution, hydroxamate formation was carried out at pH 9.0–9.2, which was somewhat higher than the optimum pH for pantothenate synthesis. At the end of incubation the enzyme action could be stopped neither by heating nor by addition of trichloroacetic acid, because the hydroxamate color obtained from the enzymatic reaction mixture seemed to be disappeared or greatly decreased by those treatments as described later[8].

The enzymatic reaction mixture cooled by the freezing mixture was kept at $-0.5°$ and pH 9.2 with the hydroxylamine reagent. Under these conditions, however, the zero-time control sometimes produced the hydroxamate color. Hence, attempts were made to avoid this color development of the zero-time control by the addition of Mn^{++} or Ba^{++} in a higher concentration to the hydroxylamine reagent as the inhibitor or the precipitator.

Addition of Ba^{++} gave the most satisfying results. As ATP was precipitated with Ba^{++}, the above results was expected from the fact

8) A. Matsuyama: unpublished data

that the enzymatic reaction system without ATP did not produce the hydroxamate color when it was incubated with the hydroxylamine reagent. Enzyme-PP which would be formed in the reaction mixture and cause the hydroxamate formation in the presence of pantoate and hydroxylamine might also be removed, because the barium salt of pyrophosphate was insoluble in the reaction mixture. Therefore, in this study the hydroxylamine reagent at pH 9.4 containing Ba^{++}, was employed for hydroxamate formation.

In Table I data are presented showing that a significant quantity of hydroxamate found when pantoate was incubated with the enzyme in the presence of ATP, using hydroxylamine as a pantoyl group acceptor. The result suggests that hydroxamate color is responsible to activation of pantoate, and activation 'of carboxyl group occurs at the expense of phosphate bond of ATP.

Anaerobical Nature of Pantoate Activation and Pantothenate Synthesis. It has been illustrated in the enzymatic reaction of the model systems concerned with amide or peptide bond formation that synthesis takes place anaerobically in the presence of ATP. The data shown in Experiment I of Table II clearly indicate that pantothenate synthesis is promoted under the anaerobical conditions. Furthermore, anaerobical nature of pantoate activation is demonstrated in Experiment II of the same table.

Proof for Involvement of -SH Enzyme. The aged enzyme preparation (15 days at 0°) responsible for the synthesis of pantothenate

TABLE I

APPEARANCE OF HYDROXAMATE IN PANTOTHENATE SYNTHESIS

The complete system contained 10 μM K-pantoate, 5 μM K-ATP, 100 μM KCl, 20 μM $MgCl_2$, 1000 μM NH_2OH, 100 μM Tris-(hydroxymethyl) aminomethane buffer (pH 8.6), and extract from 15 mg of acetone powder. Final volume 1.2 ml. Incubated at 30° for 1 hour. Zero-time control for hydroxamate determination was preincubated with Tris buffer (pH 8.6), NH_2OH and extract (NH_2OH was not used in the system "without pantoate and hydroxylamine").

	Hydroxamate found (μM)	Acid-labile phosphate* lost (μM)	Inorganic phosphate** found (μM)
Complete	0.78	2.11	2.12
Without pantoate	0.39	1.62	1.62
〃 ATP	0	0	0
〃 pantoate and hydroxylamine	0	1.48	1.46
△ due to pantoate in presence of NH_2OH	0.38	0.49	0.50

* as ATP, ** as PP

TABLE II

ANAEROBICAL NATURE OF PANTOTHENATE SYNTHESIS AND PANTOATE ACTIVATION

The complete system contained: Experiment I–10 μM K-pantoate, 10 μM β-alanine, 5 μM K-ATP, 100 μM KCl, 20 μM $MgCl_2$, 100 μM Tris buffer (pH 8.6) and extract from 13 mg of acetone powder.; Experiment II – 1000 μM hydroxylamine (pH 8.6) and all other components except β-alanine as in Experiment I. Final volume 1.0 ml (Exp. I) and 1.2 ml (Exp. II). Incubated at 30° for 1 hour.

System	Gas phase	Experiment I Pantothenate* found (μM)	Experiment II Activated pantoate found (μM)
Complete	Air	0.75	0.38
〃	N_2	1.16	—
〃	Vacuum	1.14	0.51
Without ATP	Air	0	0

* by chemical assay

from pantoate and β-alanine lost its activity. This aged enzyme was reactivated by the addition of reducing reagents such as reduced glutathione, thioglycolate and Vitamin C (Table III). Inhibition experiments with

Table III

Reactivation of Aged (15 days at 0°) Enzyme by Reducing Agents

Each tube contained the same contents as the complete system of Experiment I in Table II. Incubated at 30° for 1 hour. Pantothenate was determined by the chemical assay.

Additions	Pantothenate found (μM)
None	0.06
+5μM reduced glutathione	0.43
+5μM thioglycolate	0.29
+5μM Vitamin C	0.32

hydrogenperoxide and *p*-chloromercuribenzoate are presented in Table IV. It has been considered that hydrogen peroxide is not a specific oxidizing reagent for the -SH group, while *p*-chloromercuribenzoate is a more specific -SH reagent. The fact that inhibition of pantothenate synthesis by hydrogen peroxide is removed by thiol compounds, although it is not complete reversal, suggests the occurence of oxidation of -SH groups. The inhibition by *p*-chloromercuribenzoate and reversal of inhibition by the thiol compounds furnish strong evidence for involvement of -SH enzyme. This result was also acertained with pantothenate determination by the microbiological assay. The result obtained in the inhibition experiment with the system not containing β-alanine demonstarates that -SH enzyme may participate in pantoate activation.

DISCUSSION

It has been shown in the model synthesis of peptide or amide bonds, such as those involved in hippuric acid, glutamine, glutathion, pantothenic acid and acetyl sulfanil amide that it is the carboxyl group in common, which is activated, although these syntheses differ in form of the activated

Table IV

Inhibition of Pantothenate Synthesis and Pantoyl Hydroxamate Formation by -SH Reagents; Reversal by Reducing Reagents

The complete system was the same as that in Experiment I of Table II. Final volume 1.0ml. Incubated at 30° for 1 hour. H_2O_2 or *p*-chloromercuribenzoate in 0.5 ml Tris buffer pH 8.6 was preincubated with 0.2 ml of extract at 4° for 50 minutes, and then reducing reagents dissolved in 0.1 ml was added, if necessary, and further left for 50 minutes. The systems without reagents were similarly treated prior to incubation with the addition of water.

System	Experiment I		Experiment II	
	Pantothenate found (μM)	Activated pantoate found (μM)	Pantothenate found (μM)	
Complete	0.68	—	0.68*	0.59**
With 12 μeq. H_2O_2	0.09	—	0.09	0.07
〃 +17μM thioglycolate	0.47	—	0.51	0.43
With 0.5 M *p*-chloromercuribenzoate	0.12	—	0.16	0.14
〃 +17μM thioglycolate	0.57	—	0.40	0.45
Without β-alanine, with 1000 μM NH_2OH	0	0.37	—	—
〃, 〃, with 0.5 μM *p*-chloromercuribenzoate	—	0.03	—	—
〃, 〃, 〃 +17μM thioglycolate	—	0.39	—	—
〃, 〃, 〃 +17μM glutathione	—	0.42	—	—

* by chemical assay, ** by microbiological assay

carboxyl group[9]. In the synthesis of hippurate, CoA is required and ATP serves to form an energy-rich intermediate of benzoate by way of benzoylated CoA[10,11,12,13]. Whereas, on the other hand, it has been known that the reaction chain of acetate activation may involve ATP and CoA, and acetyl-CoA which contains a high-energy thioester bond is found to be the product of this reaction[14,15]. From equilibrium measurements the ΔF of hydrolysis of the thioester linkage of CoA was assigned to the approximate value of 10–12 Kcal. per mole[16]. CoA, by the way, seems not to be involved in the pantothenate synthesis[2,3,20]. The mechanism of pantoate activation, thereby, may be the interesting problem in view of peptide bond synthesis not requiring CoA. In this paper, the occurence of pantoate activation and the participation of -SH enzyme in the pantoate activation is illustrated. Hence, the possibility that the thiol group on the enzyme is speculated on its role as pantoyl group acceptor in pantothenate synthesis or activated pantoate attached directly to the enzyme may be in the form of the thioester linkage, appears likely. In view of these results, the pantoate activation in the presence of ATP by *E. coli* extracts is formulated as the following two-step reaction.

1) $\text{HS-E}+\text{AMP}\sim\text{PP} \rightleftarrows \text{PP}\sim\text{S-E}+\text{AMP}$
2) $\text{PP}\sim\text{S-E}+\underset{\text{pantoate}}{\text{C}_5\text{H}_{11}\text{O}_2\text{COO}^-} \rightleftarrows \underset{}{\text{C}_5\text{H}_{11}\text{O}_2\text{CO}}\sim\text{S-E}+\text{PP}$

In bacteria, acetate activation has been shown to proceed through the reaction indicated below, catalyzed by a bacterial kinase and phosphotransacetylase[16,17].

1) $\text{acetate}+\text{ATP} \rightleftarrows \text{acetylphosphate}+\text{ADP}$
2) $\text{acetylphosphate}+\text{CoA} \rightleftarrows \text{acetyl-CoA}+\text{P}$

However, in pantoate activation catalized by *E. coli* extracts the reaction sequence involving pantoate phosphorylation appears not to be the case, because of the results obtained by Maas[3]. The overall reaction of pantothenate synthesis is rather resembled to that of the acetate activation in yeast and in animal tissues. This was found as follows:

$$\text{ATP}+\text{HS}\cdot\text{CoA}+\text{acetate} \rightleftarrows \text{AMP}+\text{acetyl}\sim\text{S}\cdot\text{CoA}+\text{PP}^{[18,19]}.$$

It is noteworthy to point out in view of the transference of the energy-rich bond that the role of the thiol group on the enzyme in pantoate activation is similar to that of CoA in this reaction. However, there is apparently significant difference between these two reactions in the fine mechanism. Recently, the sequence of ATP-CoA-acetate reaction was proposed[19] as:

1) $\text{E}+\text{AMP}\sim\text{PP} \rightleftarrows \text{E}\sim\text{AMP}+\text{PP}$
2) $\text{E}\sim\text{AMP}+\text{HSCoA} \rightleftarrows \text{E}\sim\text{SCoA}+\text{AMP}$
3) $\text{E}\sim\text{SCoA}+\text{acetate} \rightleftarrows \text{E}+\text{acetyl}\sim\text{SCoA}$

Lipmann[20] mentioned about the reverse situation in pantothenate synthesis to that encountered in the ATP-CoA-acetate reaction on the basis of isotope results. The first -step in pantothenate synthesis should be an activation of the enzyme pyrophosphorylation, in contrast to the adenylation step in the ATP-CoA-acetate reaction. We do not yet understand the reason why there is difference between the modes of converting one system of bond energy to another, i.e., from P-O-P to O=C-S in the carboxyl group activation. It, however, must be noted that these reaction cycles differ in the way of formation of the enzyme-bound group in the first step, that is, enzyme-PP in pantoate activation

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and enzyme-AMP in acetate and fatty acid activation. The primary reaction involving the ATP cleavage will have to be evolved in connection with CoA requirement to clear the mechanism of the acid activating system.

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Studies on the Flavor of Green Tea

Part III. Fatty Acids in Essential Oils of Fresh Tea Leaves and Green Tea

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Fatty acids in essential oils of fresh tea-leaves (first and second essential oils of chopped-leaves and whole-leaves previously reported[1] as C–(1), C–(2), W–(1) and W–(2)) and green tea were identified by paper chromatography.

Formic, acetic, propionic *n*-butyric, isobutyric, isovaleric and caproic acid were found in C–(1) and W–(1), but in essential oil of green tea the former five acids were not to be found.

Caprylic acid was found in W–(1), W–(2) and the essential oil of green tea, but not in either C–(1) nor C–(2).

Palmitic acid was obtained in a pure form from C–(1) and W–(1).

Our studies were concerned with the fatty acids (excluding palmitic acid obtained in the pure form by standing essential oil in a deep freezer at $-24°\sim-26°C$) in essential oils of fresh tea leaves and green tea by means of paper chromatography.

EXPERIMENTAL

I. Isolation of Palmitic Acid from Essential Oils First, when essential oils from chopped-tea-leaves and whole-tea-leaves (previousely reported as C–(1) and W–(1)) were kept in a deep freezer at $-24°\sim-26°C$, there deposited a waxy substance. After filtration, it was recrystallized from ethanol. Its m.p. was $61°\sim61.5°C$ and no depression of m.p. was obserbed upon admixture with pure commercial palmitic acid.

Yield 0.12% in C–(1)
0.19% in W–(1)

II. Preparation of Samples for Paper Chromatography As for W–(1) and C–(1) after the removal of palmitic acid, they were extracted with 5% solution of sodium hydroxide of an equal volume and then washed with water. The extracts and washes were combined, acidified by adding 5 N sulfuric acid, saturated with sodium chloride and extracted with ether. After the ether extracts were dried with unhydrous sodium sulfate, ether was removed by distillation. Following this way, the acids were obtained, and were used for sampling in paper chromatography.

As for W–(2), C–(2) (second essential oil of whole- and chopped-tea-leaves) and essential oil of green tea, they were not treated and were entirely used for sampling in paper chromatography.

III. Paper Chromatography of Standard Pure Acids. One-dimentional ascending chromatography was applied using Toyo Roshi No. 50, 2×40.

Various methods of detecting acids were tried using pure commercial fatty acids, from which it was found that the method of hydrazide was suitable for $C_1\sim C_5$ acids and the method of hydroxamate suitable for $C_1\sim C_8$ acids. So, these two methods were employed for the detection of fatty acids in essential oils.

1) Hydrazide-method

Hydrazide of $C_1\sim C_5$ acids were readily soluble in water and they reduced ammoniacal solution of silver nitrate and a black color developed. They were therefore easily detected by ammoniacal solution of silver nitrate on paper.

i) Preparation of hydrazide

After an excess of ethanol was added to the acids, few drops of conc. sulfuric acid were added to them, and they were refluxed for two to four hours for esterifying. To the esters of acids thus obtained, an

1) T. Yamanishi, J. Takagaki and M. Tsujimura, This Bulletin, **20**,127 (1956)

excess of hydrazine hydrate was added, and they were then heated for eight hours. In this way, the hydrazides of acids were obtained.

ii) Detection of spots

After the developed chromatogram was dried to remove the solvent, it was sprayed with a mixture consisting of an equal volume of 10% solution of silver nitrate and 5 N ammonium hydroxide. The chromatogram was placed in an oven at 70°~80 C for five to ten minutes, and when spots appeared, the paper was moistened first with an aqueous solution of sodium chloride and subsequently with an aqueous solution of sodium hyposulfite. Then the paper was washed with water and dried. Thus, the spots were kept clear on the paper.

iii) Solvents and R_F values

Various kinds of solvents shown in Table I., were employed and pure C_1~C_6 acids and their mixtures were examined respectively, in respect of their R_F values. The result is as shown in Table I.

By this way solvent (3) was found to be most satisfactory.

TABLE I.

R_F VALUES OF HYDRAZIDES WITH VARIOUS SOLVENTS

Hydrazide \ Solvent	(1) at 20 C Lutidine 15 Isoamyl-alcohol 100 Water 10	(2) at 20 C Lutidine 10 Collidine 10 Isoamyl-alcohol 100 Water 10	(3) at 10 C Lutidine 10 Collidine 10 Isoamyl-alcohol 100 Water 10
Formic	0.09	0.07	0.11
Acetic	0.21	0.22	0.18
Propionic	0.42	0.38	0.37
n-Butyric	indistinct	indistinct	0.54
Isobutyric	0.60	0.60	0.58
n-Valeric	indistinct	indistinct	0.70
Isovaleric	indistinct	indistinct	0.70
n-Caproic	0.91	0.84	0.77

2) Hydroxamate-method

This method was found suitable for identifying C_1~C_8 acids.

i) Preparation of hydroxamate

After a suitable amount of thionylchloride was added to the acid in a test tube, the mixture was subsequently heated in an oil bath at 100°~130°C until it became almost dry. After cooling, it was

TABLE II.

R_F VALUES OF HYDROXAMATE WITH VARIOUS SOLVENTS

Hydroxamate \ Solvent*	(a) Acetic acid 1 *n*-Butanol 4 Water 5	(b) Water-saturated *n*-Butanol	(c) *tert*-Butanol 7 Formic acid 1.5 Water 1.5	(d) Water-saturated Ethyl-acetate	(e) *n*-Butanol 4.9 Acetic acid 2 Chloroform 0.1 Water 5.4	(f) Benzene 4 Acetic acid 3 Water 4	(g) *n*-Octyl-alcohol 7.5 Formic acid 2.5 Water 7.5
Formic	0.43~0.46	0.31~0.34	0.53	0.04	0.40	—	—
Acetic	0.54~0.57	0.48~0.54	0.64	0.07	0.61	—	—
Propionic	0.66~0.73	0.57~0.68	0.74	0.11	0.73	—	0.79
n-Butyric	0.82~0.83	0.74~0.79	0.83~0.87	0.43	0.74	—	0.62
Isobutyric	0.77~0.80	0.71~0.72	0.83~0.86	0.50	0.81	0.12	0.57
Isovaleric	0.83~0.89	0.83~0.84	0.87~0.90	0.80	0.83~0.84	0.46~0.49	—
Caproic	0.84~0.86	0.82~0.84	0.83	0.81	0.86	0.62~0.69	—
Caprylic	0.92~0.94	0.81~0.84	0.89~0.94	0.94	0.93	0.85~0.86	—

(*) Rate of mixture, as expressed by volume-ratio. Temperature of develpment was 12°~15°C.
Character of these solvents were as follows.
(a), (b) and (e): Suitable for C_1~C_8 acids but C_4~C_8 acids were somewhat indistinct.
(c): Exceedingly long time was required to develop, and most of the spots were found near the front.
(d): Spots tend to tail.
(f): The separatoin of caproic acid and caprylic acid was clear.
(g): Most of the spots were not distinct.

added alcoholic saturated solution of hydroxylamine hydrochloride, and thereupon in order to give it a weak alkaline reaction, 5% alcoholic solution of sodium hydroxide was further added. Then it was heated at 100°C for five to ten minutes.

ii) Detection of spots

After the developed chromatogram was dried, it was sprayed with 10% alcoholic solution of ferric chloride. Immediately after there appeared purple or purplish brown spots on the light-yellow ground.

iii) Solvents and R_F values

Various solvents as listed in Table II., were used. The R_F values by use of these solvents are shown in Table II.

IV. Identification of Acids in Essential Oils by Paper Chromatography Acids of C–(1) and W–(1) prepared as mentioned above and whole essential oil of C–(2), W–(2) and green-tea were subjected to paper chromatography in the same way as in the case of pure acids. R_F values were compared with those of the pure acids developed at the same time.

The acids of essential oils identified in this way are shown in Table III.

Table III.

Acids in Essential Oils Identified by Papar Chromatography

Acid identified \ Sample	C–(1)	W–(1)	C–(2)	W–(2)	Green tea
Formic	+	++	−	+	−
Acetic	++	++	+	−	−
Propionic	+	±	−	−	−
n-Butyric	+	±	−	−	−
Isobutyric	+	±	−	−	−
Isovaleric	+	±	−	−	+
Caproic	++	++	+	−	+
Caprylic	−	+	−	+	+

Mark ++, +, ±, − denote the grade of size and color-intensity of spots.
++...large and deep color
+...normal
±...somewhat doubtful
−...non-spot

The presence of acetic, propionic, *n*-butyric, isobutyric, isovaleric, caproic and caprylic acids was found to agree with Takei's report[2].

From the result shown in Table III., it is presumed that in the process of steaming, the following acids namely, formic, acetic, propionic, isobutyric and *n*-butyric probably flee from the tea leaves or change into a certain compound, while on the other hand caprylic acid is newly produced.

SUMMARY

(1) Fatty acids in essential oils of tea leaves (four kinds of essential oils previously reported, are represented as C–(1), W–(1), C–(2) and W–(2)) and green tea were identified by paper chromatography.

(i) Acetic, propionic, *n*-butyric, isobutyric, isovaleric and caproic acid were found in C–(1) and W–(1). These acids have been reported by Takei et al.

(ii) Caprylic acid was found in W–(1), W–(2) and in the essential oil of green tea, but not in C–(1) and C–(2).

(iii) Formic acid was found in C–(1), W–(1) and W–(2). The presence of this acid in fresh tea-leaves has not been reported previously.

(2) Palmitic acid was obtained in a pure form from C–(1) and W–(1), by standing at −24°～−26°C.

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[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 1, p. 58~61, 1957]

Studies on Amino Acid. III. On the Synthesis of DL-Tryptophan*

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The synthetic procedure for the preparation of DL-tryptophan, reported by Warner and Moe has been investigated. In the Michael-type condensation between acrolein and ethyl acetamidomalonate, a number of organic and inorganic basic substances were employed and their catalytic activity was studied. Anion exchange resins were found to be a convenient catalyst for the reaction. γ-Acetamido-γ, γ-dicarbethoxybutyraldehyde, a product of the above reaction, was obtained as phenylhydrazone, and the Fischer indole synthesis, that is, the conversion of the phenylhydrazone into ethyl α-acetamido-α-carbethoxy-β-(3-indole)-propionate, was successfully carried out by employing cation exchange resins as a catalyst. Subsequent hydrolysis and decarboxylation of the indole derivative gave DL-tryptophan in a good yield.

Since the first success of the synthesis of DL-tryptophan by Ellinger in 1907, only a few methods had been reported before the second World War. However, with the remarkable progress of amino acids syntheses in recent years, a number of preparative methods of DL-tryptophan have appeared.

Of the hitherto reported methods, the synthetic route employed by Warner and Moe[1] appears to be a convenient one. In this procedure shown in Table I, they used a small amount of sodium alcoxide as a catalyst for the addition reaction of ethyl acetamidomalonate to acrolein and obtained the product as its phenylhydrazone (III) in a 65–87% yield. In this Michael-type addition reaction, the authors employed various organic and, inorganic bases and also strong base anion exchangers to investigate their catalytic activity. The results obtained are summarized in Table II. Of the inorganic basic substances studied, caustic alkalis, alkali carbonates, alkali bicarbonates and alkali earth hydroxide could accelerate the reaction. The yields of the phenylhydrazone fell with the lowering of basicity of the catalysts used. Namely, the use of 0.05 mol. of caustic alkali is enough as catalyst, however, 0.1 mol. and 1 mol. of sodium carbonate and sodium bicarbonate, respectively, are necessary to attain the reaction. It is of interest to note that this is the first occasion in which powdered caustic alkalis, alkali carbonates and alkali bicarbonates in benzene were found to be effective catalysts for the Michael reaction. When a little amount of hydroquinone was added to prevent polymerization of acrolein in this reaction, the yield lowered or the reaction did not proceed at all. So it is assumud that hydroquinone is a negative catalyst of the reaction under the conditions employed by the authors. Of the organic bases studied diethylamine and triethylamine gave the best yield, while dimethylaniline and pyridine showed no catalitic activity. Generally speaking, it appears that effective catalysts for this reaction should have basicity more than approximately 10^{-5} in the dissociation constant. Through the experiments, diethylamine and triethyla-

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1) D.T. Warner and O.A. Moe, *J. Am. Chem. Soc.*, **70**, 2763 (1948); *ibid.* 2765

TABLE I

SYNTHETIC ROUTE OF DL-TRYPTOPHAN

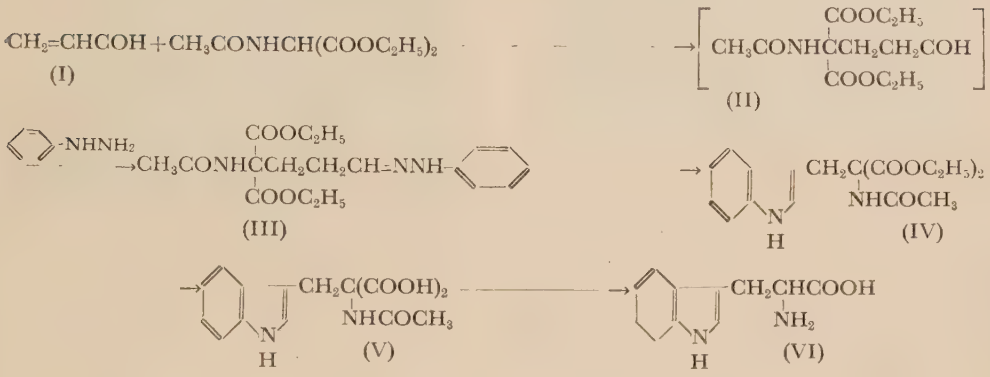
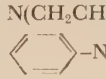
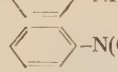


TABLE II

EFFECT OF CATALYST ON THE ADDITION REACTION

Catalyst	mol.	Phenylhydrazone (III, crude) Yield (%)	m.p (°C)	Catalyst	mol.	Phenylhydrazone (III, crude) Yield (%)	m.p. (°C)
CH_3ONa	0.1	63.6	137–41	$(Et)_2NH$	0.1	77.0	125–33
〃 (H.Q.)	0.1	23.4	135–41	$(Et)_3N$	0.1	75.0	138–41
KOH	0.1	72.5	139–41		0.1	57.0	138–41
〃	0.05	65.0	141–42		0.1	31.6	137–41
〃 (H.Q.)	0.05	0		Triton B	0.1	55.5	135–38
NaOH	0.05	68.2	140–43	$NH_2CH_2CH_2OH$	0.1	13.4	136–39
K_2CO_3	0.1	63.5	130–35	$NH(CH_2CH_2OH)_2$	0.1	0	
Na_2CO_3	0.1	57.4	135–40	$N(CH_2CH_2OH)_3$	0.1	0	
〃	0.05	0			0.1	0	
$NaHCO_3$	1.0	38.0	130–40		0.1	0	
〃	0.1	0		Pyridine	0.1	0	
NaCN	0.1	67.0	139–43	2-Aminopyridine	0.1	43.2	135–39
$Ba(OH)_2$	0.1	65.0	137–40	2-Aminothiazole	0.1	29.3	135–36
$NaNH_2$	0.05	53.6	137–40	2-Aminopyrimidine	0.1	0	
NH_4OH	0.1	0		Amberlite IRA–400(OH)	+	62.0	136–39
$(NH_4)_2CO_3$	0.1	0		〃		20.0	134–37
CH_3COONa	0.1	0		Amberlite IRA–400(CN)	+	69.0	137–41
				〃		23.5	137–41

(H.Q.): hydroquinone
+ Alcohol was used as solvent.

mine gave the best yield, and caustic alkalis followed, although, it was observed that the use of caustic alkali is advantageous over the former amines in regard to the melting point and coloring of the phenylhydrazone obtained. According to the results obtained by the authors, caustic alkali gave better results than the sodium alcoxide of the original report.

The reaction was also carried out by employing strong basic anion exchangers, Amberlite IRA-400, and a satisfactory result was obtained as previously reported by the authors[2]. The resins in both hydroxide-form and cyanide-form prepared by the previously reported method were found effective as catalyst.

The phenylhydrazone of γ-acetamido-γ, γ-dicarbethoxybutyraldehyde obtained by the above reaction, was converted to ethyl α-acetamido-α-carbethoxy-β-(3-indole)-propionate (IV). This Fischer indole synthesis, was carried out by sulfuric acid in the original report. Instead of this conventional catalyst, the authors studied the employment of ion exchangers for the conversion of various phenylhydrazones including (III) into indole derivatives, the results were reported in the other paper[2]. According to this method, ring-closure took place in a good yield by refluxing the phenylhydrazone with sulfonic acid resins in aqueous solution. The procedure is simple and gives a more pure product, and the used resins can be used for successive runs.

The product (IV), obtained by this method was identified with the authentic sample prepared by the method of Howe and Zambito[3]. Subsequent hydrolysis of the product by the method reported by Snyder and Smith[4] gave DL-tryptophan in a satisfactory yield.

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3) E.E. Howe, A.J. Zambito, H.R. Snyder, and M. Tishler, *J. Am. Chem. Soc.*, **67**, 38 (1945).

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EXPERIMENTAL

γ-Acetamido-γ, γ-dicarbethoxybutyraldehyde Phenylhydrazone (III). *a) The method employing organic and inorganic bases:* To a suspension of ethyl acetamidomalonate (8.7 g) and the catalyst (0.05–0.1 mol.) in benzene (16 ml), acrolein (3 ml) diluted with benzene was added dropwise under stirring. The reaction was usually exothermic and kept below 40°. After the addition of acrolein solution, stirring was continued for 2 hours at room temperature. The reaction mixture became almost clear at the end of the reaction. The insoluble material was removed by filtration if necessary. To the filtrate acetic acid (2 ml) and phenylhydrazine (4.3 g) were added and heated at 50° for a while. The crude phenylhydrazone (III) separated by cooling the above solution. Recrystallization from 80% aqueous alcohol gave colorless prisms, m.p. 144–145°. *Anal.* Found: C, 59.17; H, 7.11; N, 11.83. Calcd. for $C_{18}H_{25}O_5N_2$: C, 59.55; H, 6.94; N, 11.56.

b). The method employing anion exchangers: To a stirred mixture of ethyl acetamidomalonate (4.4 g) in ethyl alcohol (10 ml) and Amberlite IRA–400 CN-form (2.2 g), acrolein (1.6 ml) was added dropwise at 5°. After further stirring below 10° for 2 hours, the resins were filtered off and washed with a little amount of ethyl alcohol. To the combined filtrate and washings, acetic acid (1 ml) and phenylhydrazine (2.4 g) were added and heated to 50°. Addition of water (10 ml) and chilling gave the phenylhydrazone, yield 5.2 g (69%). A mixed melting point determination with the authentic sample showed no depression.

Ethyl α-Acetamido-α-carbethoxy-β-(3-indole)-propionate (IV). The phenylhydrazone (III) was refluxed with Amberlite IR–120 in an aqueous solvent as described in the previous report. The indole derivative (IV) was obtained in 70–75%, m.p. 161–162°.

The product showed no melting point depression upon admixture with the sample prepared by the method of Howe and Zambito[3].

α-Amino-β-(3-indole) propionic Acid (DL-Tryptophan) (V). The indole derivative (IV) (3.4 g) was heated with 50% sodium hydroxide solution (4 ml). The crystals disappeared and resulted a clear solution during the reaction. Refluxing was continued for about 3 hours and it was decolorized, then hydrochloric acid was added below 10°. The separated crude α-acetamido-α-carboxy-β-(3-indole)-propionic acid (V) was filtered and then washed with water. The four-fifth of the dried product (V) was boiled

with water (12 ml) for 2.5 hours, then 25% sodium hydroxide solution (5 ml) was added and refluxing was continued for 20 hours. After decolorization, the solution was neutralized with acetic acid (2.4 g) separating white precipitate. After standing over-night, the separated precipitates were collected and washed with water and alcohol. The obtained crude DL-tryptophan contaminated with inorganic salt, was dissolved in 2.5% sodium hydroxide solution. Addition of acetic acid (0.8 ml) gave white minor crystals (1.7 g). Recrystallization from 20% aqueous alcohol yielded bright white scales, m.p. 280–285° (dec.), yield 1.3 g (81.2%). *Anal.* Found: C, 64.63; H, 5.77, N, 14.09. Calcd. for $C_{11}H_{12}ON_2$: C, 64.69; H, 5.92; N, 13.73.

Acknowledgement. The authors grateful to Prof. S. Sugasawa of Tokyo University, Prof. H. Mitsuda of Kyoto University and Dr. Fujisawa, Director of this Laboratory, for their helpful advice and encouragement given in the course of this study.

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Studies on Amino Acids. IV. Studies on the Enzymatic Resolution (III): Enzymatic Resolution of DL-Tryptophan.*

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Received September 19, 1956

To find out a convenient method for the resolution of DL-tryptophan, asymmetric synthesis of acyl L-tryptophan anilides, and asymmetric hydrolysis of DL-tryptophan esters and DL-tryptophan amide were investigated. As a result, the enzymatic hydrolysis of DL-tryptophan amide was found to be an advantageous procedure for the resolution of DL-tryptophan.

As a part of the studies on amino acids, the authors have been studying the enzymatic resolution of racemic amino acids, and the results of the investigations on the enzymatic resolution of DL-methionine[1] and DL-lysine[2] have been already reported by the authors. This paper presents the results of the study on the resolution of DL-tryptophan.

Several reports on the asymmetric synthesis of acyl L-tryptophan anilide from acyl DL-tryptophan and aniline by papain have been already published (Table I). The authors carried out the experiment by modifying the method reported by Albertson. As a result, asymmetric synthesis was attained and acetyl L-tryptophan anilide was obtained in an almost quantitative yield. However, the following hydrolysis of acetyl L-tryptophan anilide to L-tryptophan was unsuccessful under the conditions employed by the authors. So, it may be possible to say that the resolution procedure through the enzymatic anilide synthesis is not adequate for the preparation of L-tryptophan.

On the enzymatic resolution of tryptophan esters, Brenner[6] studied the hydrolysis of the methyl ester and reported that pure L-tryptophan was obtained when crystalline chymotrypsin was employed. As the employment of amorphous chymotrypsin or pancreatin resulted the product of optically lower purity, so the purification procedure through naphthalene-β-sulfonates of the crude isomers was necessary to obtain a pure product. The authors also investigated the hydrolysis of the methyl ester by dissolving the ester in benzene so as to prevent chemical hydrolysis during the enzymatic reaction, and the benzene layer was contacted with an aqueous extract of pancreas enzyme preparation with stirring. However, the optical purity of the hydrolyzed product was somewhat low, namely about 80%. Whereas, on the other hand, Ravinovich[7] reported the resolution through the enzymatic hydrolysis of the isopropyl ester. The isopropyl ester was therefore hydrolyzed as in the case of the methyl ester. Although the product obtained by this procedure was optically pure, the yield of purified L-tryptophan was not satisfactory. These results are listed in Table II.

As the above described procedures were

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** Honjo-kawasaki-cho, Oyodo-ku, Osaka.

1) S. Yamada, I. Chibata and S. Yamada, *J. Pharm. Soc. Japan.* **75**, 113 (1955).

2) I. Chibata, S. Yamada and S. Yamada, This Bulletin, **20**, 174 (1956.)

3) D.G. Doherty and E.A. Propenoe, *J. Biol. Chem.*, **189**, 447 (1951).

4) N.F. Albertson, *J. Am. Chem. Soc.*, **73**, 452 (1951).

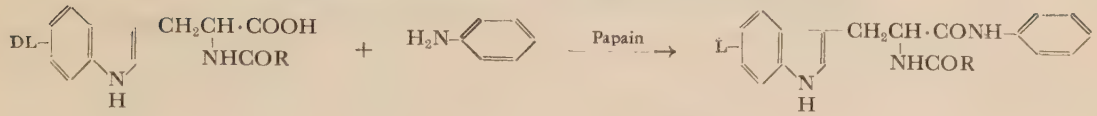
5) H.T. Hanson and E. Smith, *J. Biol. Chem.* **179**, 815 (1949).

6) M. Brenner, *Helv. Chim. Acta.*, **31**. 1908 (1948).

7) P.N. Rabinovich, *Daklady Akad. Nauk S.S.S.R.*, **85**, 117 (1952); C.A., **47**, 8015 (1953).

TABLE I

ASYMMETRIC SYNTHESIS OF ACYL L-TRYPTOPHAN ANILIDES

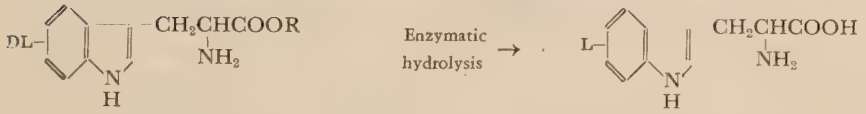


Acyl	L-Anilide (yield)	Ref.
Acetyl	90%	3
Acetyl	77%	4
Acetyl	92%	*
Carbobenzoxy	100%	5

* Result obtained by the authors.

TABLE II

ASYMMETRIC HYDROLYSIS OF DL-TRYPTOPHAN ESTERS



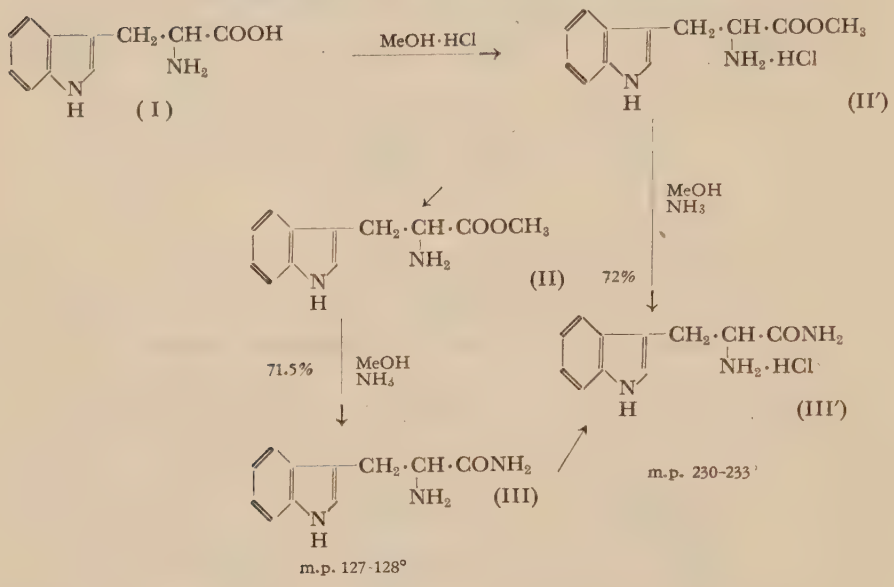
Ester	Enzyme Preparation	L-Tryptophan yield (%)	$[\alpha]_D$	Ref.
Methyl	Cryst. Chymotrypsin	crude 96%	$-30 \pm 2°$	6
〃	Pancreas Enz.	crude 90%	$-23°$	〃
	(naph-β-sulf.)†	51%	$-32.5 \pm 1°$	〃
〃	Pancreatin	crude 68%		*
		recryst. 52%	$-25.9°$	
i-Propyl	Pancreatin	90%	$-31.6°$	7
〃	〃	crude 60.3%		*
		recryst. 39.6%	$-32.5°$	

* Result obtained by the authors. † Purified as naphthalene-β-sulfonate.

not so satisfactory for the resolution of DL-tryptophan, the authors synthesized a new tryptophan derivative, DL-tryptophan amide, and studied the enzymatic hydrolysis of this substrate. The substrate was prepared from DL-tryptophan by esterification and subsequent amination as shown in Table III. For the hydrolysis of the substrate, enzyme preparation such as pancreatic enzyme, "Protamylase", Aspergillus enzyme "Taka-diastase" and papain were employed and their susceptibility and the optimal pH were investigated. In this experiment, the extent of hydrolysis was estimated by the titration of liberated carboxyl groups according to the Grassman-Hyde[8] procedure. As a result, it was found that the amide was asymmetrically hydrolyzed most rapidly by pancreas enzyme, and the optimal pH for Protamylase, papain and Taka-diastase was approximately 5.5 and 7, respectively. So, the practical resolution of DL-tryptophan amide was carried out by the employment of pancreas enzyme. After the completion of enzymatic reaction, organic solvent-soluble D-tryptophan ester was separated from insoluble L-tryptophan by extraction. The L-tryptophan obtained by this procedure showed high optical purity, and furthermore, the yield was satisfactory. The influence of several metal ions to this enzyme reaction was also studied. The study revealed that, Mn^{++} and Mg^{++} activate the enzyme, while Co^{++} retards the reaction. This metal activation is useful for the practical resolution in

8) W. Grassmann and W. Hyde, *Z. physiol. Chem.*, **183**, 32 (1929).

TABLE III
SYNTHETIC ROUTE OF DL-TRYPTOPHAN AMIDE



the shortening of incubation and the curtail of enzyme preparation.

The procedure above described through the enzymatic hydrolysis of DL-tryptophan amide can be said to be an advantageous method for the preparation of L-tryptophan. That is, the substrate is readily hydrolyzed by a small amount of easily available pancreas enzyme preparation and optically pure L-tryptophan is obtained in a good yield.

EXPERIMENTAL

Asymmetric Synthesis of Acetyl-L-Tryptophan Anilide. In an Erlenmeyer flask was placed 4.92 g of acetyl-DL-tryptophan, 20 ml of N-NaOH, 2 ml of aniline and 20 ml of citrate buffer (pH 4). The enzyme solution, prepared by extracting 1.2 g of papain with 15 ml of distilled water containing a small amount of potassium cyanide, was then added. The pH was brought up to 4.5 if necessary, and the flask was stoppered and placed in an incubator at 37° for five days. After cooling the mixture, the separated anilide was filtered and washed with water; yield 2.92 g (92%), m.p. 196–199°, $[\alpha]_D^{28}$ +66.9° (5% in acetic acid).

No way could be found to prepare L-tryptophan from this compound by acid hydrolysis without extensive destruction.

Asymmetric Hydrolysis of DL-Tryptophan Esters. *i) Enzymatic Hydrolysis of the Methyl Ester.* To a solution of 2.7 g of DL-tryptophan methyl ester dissolved in 20 ml of benzene, 5 ml of 5% pancreatin extract was added and it was then stirred at 37° for 7 hours. After the reaction, the separated crystals were collected by filtration and washed with alcohol; yield 0.85 g (67.2%). Recrystallization of the crude L-tryptophan from aqueous alcohol gave the product, m.p. 272–275° (dec.), $[\alpha]_D^{20}$ −25.9° (1% in water).

ii) Enzymatic Hydrolysis of the Isopropyl Ester. A mixture of 4.1 g of DL-tryptophan and 30 ml of isopropyl alcohol containing 15% hydrogen chloride was refluxed for one hour. After cooling, the separated crystals of DL-tryptophan isopropyl ester hydrochloride were filtered and washed with ether; yield 4.0 g (70.5%), m.p. 227–228° (dec.). A mixture of DL-tryptophan isopropyl ester hydrochloride dissolved in 10 ml of water and 30 ml of ether was cooled to −10°, and aqueous ammonium hydroxide was added under stirring until it became alkaline to phenolphthalein. The ethereal layer was separated and the aqueous layer was repeatedly extracted with ether. The combined ether extracts were dried over anhydrous sodium sulfate. Evaporation of the solvent left the isopropyl

ester, yellow oil; yield 1.4 g (80.5%).

The ester thus obtained, was dissolved in 20 ml of benzene and 5 ml of 5% extract of pancreatin was added. The reaction mixture was stirred at 37° for 8 hours. After completion of the reaction, the separated crude L-tryptophan was collected; yield 0.35 g (60.3%). Recrystallization from aqueous alcohol yielded a pure product, 0.23 g (39.6%), m.p. 277–278° (dec.), $[\alpha]_D^{20}$ −32.5° (1% in water).

Asymmetric Hydrolysis of DL-Tryptophan Amide. *i) Preparation of DL-Tryptophan Amide.* To a stirred suspension of 5 g of DL-tryptophan methyl ester hydrochloride in 80 ml of ether, aqueous solution containing 5.4 g of potassium carbonate was added under cooling in an ice-salt bath. The ethereal layer and the ether extract of the aqueous layer were combined, washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent left 4 g of the oily free ester. The ester was dissolved in 60 ml of methanol saturated with ammonia and allowed to stand for several days in a stoppered pressure bottle at room temperature. Concentration of the reaction mixture and recrystallizations from methanol-chloroform and water gave DL-tryptophan amide, colorless pillars, m.p. 127–128°, yield 2.8 g (71.5%).

Anal. Found: C, 65.46; H, 6.46; N, 20.65. Calcd. for $C_{11}H_{13}ON_3$: C, 65.00; H, 6.45; N, 20.68.

The amide was dissolved in methanol and dry hydrogen chloride was introduced. Concentration and recrystallization from methanol-chloroform gave the hydrochloride, m.p. 231°.

Anal. Found: C, 54.70; H, 5.48; N, 18.01. Calcd. for $C_{11}H_{14}ON_3Cl$: C, 55.11; H, 5.89; N, 17.53.

The hydrochloride of DL-tryptophan amide was also synthesized from the ester hydrochloride as follows: 5 g of DL-tryptophan methyl ester hydrochloride was dissolved in 70 ml of methanol saturated with ammonia. The reaction mixture was allowed to stand for several days in a stoppered pressure bottle at room temperature. Then the mixture was concentrated in vacuo, the residue recrystallized from methanol-chloroform yielding DL-tryptophan amide hydrochloride; yield 3.4 g (72.0%), m.p. 230–233°. A mixed melting point determination with the sample obtained from the former method showed no depression.

ii) Optimal pH and Enzyme Activity. Susceptibility of the substrate and the optimal pH to the various enzyme preparations such as Protamylase (Teikoku Hormone Mfg. Co.), Taka-diastase (Sankyo Co. Ltd.) and Papain (E. Merck Darmstadt) were investigated under comparable conditions. Namely, to 2 ml of 0.25 M neutralized DL-tryptophan amide hydrochloride solution, 7.5 ml of Veronal buffer in the range of pH 4–9 and 0.5 ml of 10% extract of the enzyme preparation were added. The digestion solution was incubated at 37° for 20 hours and the extent of the reaction was assayed by titration. The results are shown in Fig. 1.

Although all the enzymes tested could hydrolyze the substrate, pancreatic enzyme, Protamylase, was most active. The optimal pH for the action of Protamylase, papain and Taka-diastase was found to be about 8.0, 5.5 and 7.0, respectively. In a longer incubation of the digestion mixture of Protamylase, hydrolysis did not proceed over 100% (50% of the racemic substrate), which indicates the action of pancreas enzyme to the substrate is asymmetric.

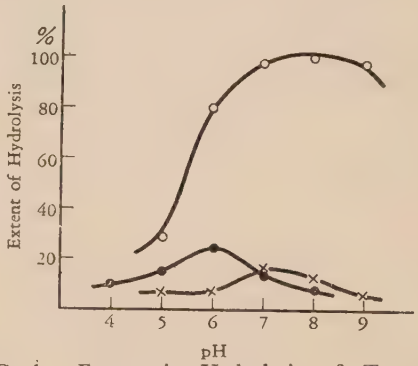


FIG. 1. Enzymatic Hydrolysis of Tryptophan Amide.

○—○ Protamylase. ●—● Papain.
×—× Taka-diastase.

iii) Isolation of L-Tryptophan. To a solution containing 2.4g of DL-tryptophan amide hydrochloride, 10ml of 5% extract of Protamylase was added. The solution was adjusted to pH 8 and filled up to 100 ml with water and/or buffer solution. Incubation was carried out at 37° and the extent of hydrolysis was checked during the reaction. The reaction was usually attained within 70 hours. After the reaction, the incubation mixture was made up to pH 5 with acetic acid and boiled for a while to coagulate the enzyme protein. The separated precipitate was removed and the filtrate was concentrated in vacuo. To the residue 100 ml of alcohol was added and the mixture was allowed to stand overnight in a refrigerator. The separated crude L-tryptophan was collected by filtration and recrystallized from aqueous alcohol; it yielded

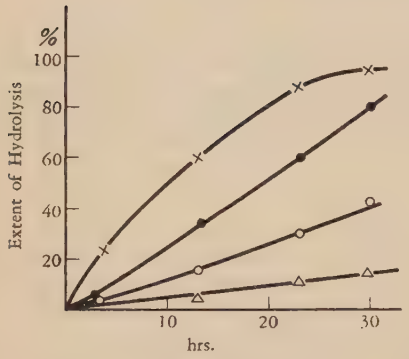


FIG. 2. Effect of Metal Ions to the Enzymatic Hydrolysis of Tryptophan Amide.

○—○ Control (no addition). ×—× Mn^{++}
●—● Mg^{++} △—△ Co^{++}

colorless scales of L-tryptophan; yield 0.72 g (70.2%), m.p. 284–287°, $[\alpha]_D^{23}$ −32.0° (0.5% in water).

Anal. Found: C, 64.72; H, 6.03; N, 13.46. Calcd. for $C_{11}H_{12}ON_2$: C, 64.69; H, 5.92; N, 13.73.

iv) *Effect of Metals.* The effect of certain divalent metal ions was studied by the addition to the reaction solution. Each reaction solution consisted of 2 ml of 0.25 M neutralized DL-tryptophan amide hydrochloride solution, 0.5 ml of 2.5% extract of Protamylase, 4.5 ml of distilled water, 1ml of 0.005 M metal salt solution, and 2 ml of Veronal buffer at pH 8.0. Ions of manganese and magnesium were found to stimulate the enzyme reaction (Fig. 2). Besides these ions, salts of cobalt and strontium were also tested and it was proved that cobalt was inhibitory while strontium showed no effect toward the enzyme. It was found that by adding the proper metal ion to activate the enzyme reaction, incubation of the practical resolution procedure is either shortened or quantity of the enzyme preparation employed can be decreased.

Acknowledgement. The authors are grateful to Prof. H. Mitsuda of Kyoto University and Dr. M. Fujisawa, Director of this Laboratory, for their helpful advice and encouragement.

Isolation of Fumaryl-pyruvic Acid as an intermediate of the Gentisic acid Oxidation by *Pseudomonas ovalis* var. *S-5*

Sir:

In a previous paper[1], we presumed that the initial intermediate after the oxidative ring cleavage of gentisic acid by *Ps. ovalis S-5* might be maleyl-pyruvic acid or fumaryl-pyruvic acid.

Recently, we have succeeded in isolating one of the suggested intermediates in a crystalline form. (Fig. 1)

The crude extracts of *m*-hydroxybenzoate

FIG. 1.

induced cells of *Ps. ovalis S-5*, prepared as described elsewhere[2], were dialyzed against distilled water.

When the dialyzed crude extracts were incubated with sodium gentisate, an *o*-phenylenediamine positive β-diketo substance was accumulated. Such accumulation of the β-diketo substance was not detected when the non-dialyzed crude extracts were employed.

After sodium gentisate was incubated at 30°C. for 40 min. with the dialyzed extracts, the reaction mixture was deproteinized with meta-phosphoric acid and continuously extracted with peroxide free ether. Ether was removed *in vacuo* and a yellow substance was obtained. From this yellow substance the β-diketo intermediate was obtained in a crystalline form by several recrystallizations from ethylacetate-petroleum ether and ethanol-benzene.

The crystalline substance was readily hydrolyzed by the non-dialyzed crude extracts of our *Pseudomonas* cells and also by the acylpyruvase from rat liver[3], forming two acids, i.e., fumaric and pyruvic acids. These two acids were detected and identified by paper chromatography. However, maleic acid was not detected. The formation of a dicarboxylic acid as the intermediate during gentisic acid oxidation was also observed manometrically.

Analytical data of the crystalline intermediate were as follows:
(d.p. 180–181°C) *Anal.* Calcd. for $C_7H_6O_6$: C, 45.17; H, 3.25, Found: C, 45.20; H, 3.40.

This substance readily reacted with carbonyl reagents such as sodium niroprusside,

1) Arima, K., et al. This Bulletin **19**, 51 (1955).

2) Arima, K., et al. Proceedings of the 30th Anniversary of the Japanese Biochemical Soceity P. 43-44 (1956)

3) Meister, A. & Greenstein, J.P., *J. Biol. Chem.* **175**, 573 (1948)

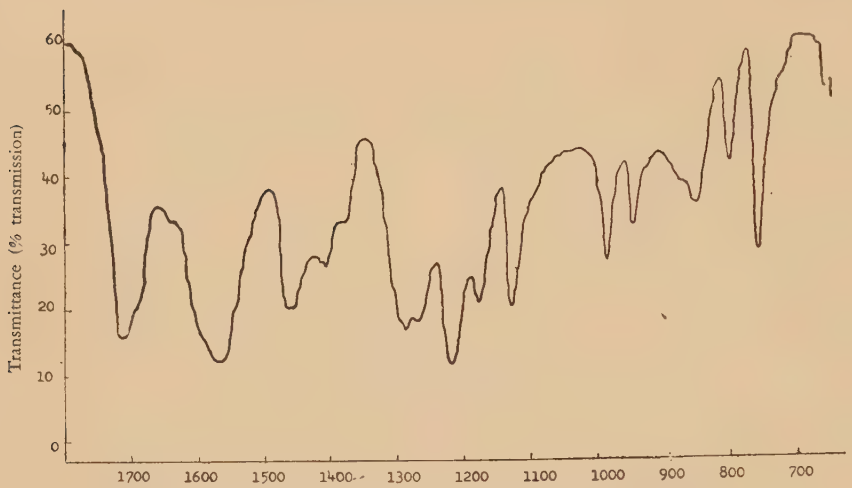


FIG. 2. Infra-red Spectrum of Fumaryl-pyruvic acid in Nujol Mull. Frequency (cm^{-1})

2,4-dinitrophenyl-hydrazine, and semicarbazide. The reaction with *o*-phenylenediamine was also strongly positive.

The UV-absorption spectra of the crystalline intermediate showed the maximum at 335 mμ (ε=8300) in 0.1 N HCl; at 340 mμ (ε=9400) in phosphate buffer pH 7.3; and at 350 mμ (ε=10300) in 0.1 N NaOH.

The infra-red spectra in Nujol mull (Fig. 2) showed the presence of monoenol-β-diketone (1560–1570 cm^{-1}); α, β-unsaturated acid HOOC–C=C (1710 cm^{-1}); and –C=C– (H H, H H) *trans* (980 cm^{-1}).

All the facts observed for the crystalline intermediate are compatible with the structure (Fig. 3, III) of fumalyl-pyruvic acid. Therefore, the gentisic acid oxidation by *Pseudomonas ovalis S-5* is demonstrated in the following scheme:

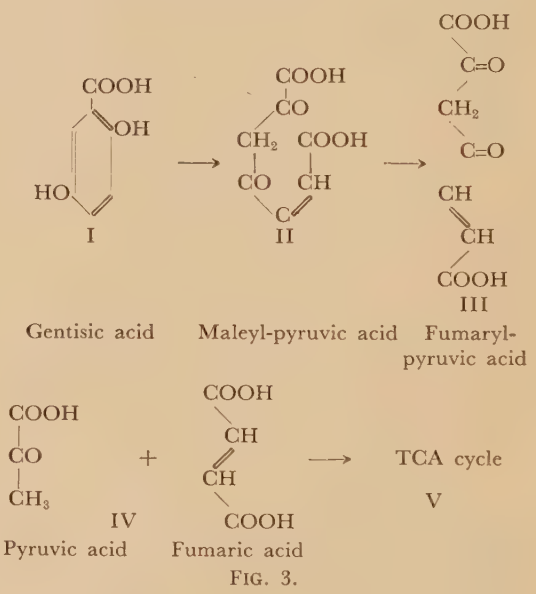


FIG. 3.

The authors wish to express their appreciation to Professor Kin-ichiro Sakaguchi of Univ. of Tokyo for his guidance and encouragement, to Assist. Prof. Takehiko Shimanouchi for his instruction on the infra-red analysis and to the members of Professor Sumiki's laboratory for their helpful advice. They are also deeply indebted to the Central Institute of the Japan Monopoly Corporation for taking the infra-red spectrum given here.

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Fermentative Production of L-Glutamic Acid from α-Ketoglutaric Acid and Ammonium Salt*

Sir:

Up to the present, L-glutamic acid has been produced by the hydrolysis of protein materials or by the chemical synthesis. A new practical method for the preparation of L-glutamic acid was devised. This method is comprised of the incubation of the living cells of microorganism in the medium A_3 which contains α-ketoglutarate, an ammonium salt, minerals, and growth factors.

Searches for the microorganisms which are able to grow in the medium A_3 and accumulate L-glutamate in the medium were carried out. The accumulation of L-glutamate was detected in the cultures of the microorganisms belonging to the following genera: *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Bacterium*, *Erwinia*, *Escherichia*, *Micrococcus*, *Serratia*, *Pseudomonas*, *Xanthomonas*, *Debaryomyces*, *Hansenula*, *Mycotorula*, *Pseudosaccharomyces*, *Saccharomyces*, *Willia*, *Aspergillus*, *Penicillium* and *Rhizopus*.

Strains belonging to the Genus *Pseudomonas* were isolated more frequently than the others, and were proved to have a powerful ability to accumulate L-glutamate. A strain of *Pseudomonas ovalis*, which showed the highest level of L-glutamate accumulation, was used in the following experiments.

Investigation of the effects of nitrogen sources on the L-glutamate accumulation revealed that ammonium chloride was the best nitrogen source for L-glutamate accumulation.

It was found that the L-glutamate accumulation was dependent on the surface-volume ratio of the liquid medium in stationary culture. The larger the surface-volume ratio, the smaller was the amount of L-glutamate. Although the aerobic condition was favourable for the growth of this organism, no accumlation of L-glutamate took place under strong aerobic conditions such as shake culture. Under the anaerobic condition both the growth of the organism and the accumulation of L-glutamate were slow, but the amount of L-glutamate increased within ten days' incubation. These facts suggested that the consumption of L-glutamate was prevented by the anaerobic condition.

The accumulation process of L-glutamate was, therefore, separated from the growth-phase of the organism. After the organism was grown in the medium A_3 on a shaker at 30°C for 17 to 18 hrs., the cells were harvested by centrifugation and washed thrice. The washed cells were added to the fresh medium A_3, and incubated at 30°C.

The highest yield of L-glutamate was obtained when the pH of the medium was maintained neutral or slightly alkaline and the incubation was carried out anaerobically. Within 24 to 30 hrs., about 98% of the added α-ketoglutarate was consumed and 30 μM L-

TABLE I

THE ACCUMULATION OF L-GLUTAMATE AFTER 24 HRS'. INCUBATION

Conditions of incubation	Initial pH	α-K-G consumed	L-glu. accumulated
Incubated in the air.	7.2**	31.2 μM/ml	12.5 μM/ml
	7.4*	53.0	23.6
	8.6*	52.6	26.1
Incubated in vacuo.	7.2**	20.2	9.4
	7.4*	52.7	32.2
	8.6*	53.1	28.9

α-K-G = α-ketoglutarate
L-glu. = L-glutamate
The initial concentration of α-K-G was 54.0 μM/ml.
The initial concentration of the cells was 30 mg cells (wet weight)/ml.
** The pH of the medium was not adjusted.
* The pH of the medium was adjusted to its initial pH at the intervals of 6 hrs.

* This work was presented at the meeting of the Institute for Food Technology, Aichi Prefecture, held on August 1, 1956.

glutamate per ml, an amount corresponding to 60% of the consumed α-ketoglutarate, was accumulated in the medium. When the pH was not controlled, even though it started at pH 7, the amount of L-glutamate accumulated was only 1/3 that of the above condition (Table I).

Attempts to clarify the mechanisms of L-glutamate accumulation are being carried out.

The authors wish to express their thanks to Assist. Prof. H. Iizuka and Mr. K. Komagata for identifying the microorganisms used in this study, and to the Ajinomoto Co. Ltd. for supplying the chemicals.

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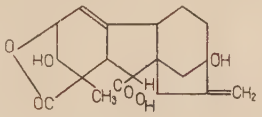
Chemical Structure of Gibberellins. VII.

(Biochemistry of Bakanae Fungus. Part 37)

Sirs:

Gibberellins A_1, A_2 and gibberellic acid (our gibberellin A_3 and Dr. Stodola's gibberellin X) were isolated and characterized as previously reported[1]. We proposed the formula $C_{20}H_{28}O_7$ for G A_2 (gibberellin A_2). However, from the analytical data of its derivatives and degradation products and the determination of crystalline water of G A_2 methyl ester by Karl Fisher's method, we correct its molecular formula as $C_{19}H_{26}O_6$. Cross et al.[2] proposed (I) as a tentative structure of G acid.



G A_1 and G A_2 have one carboxyl (methylation and titration) and one lactone group (A_1 : 1763 cm^{-1}, A_2 : 1755 cm^{-1} in infra-red spectra). G A_1 methyl ester gives a mixture of monoacetyl and diacetyl derivatives on acetylation. One of these two hydroxyl groups is secondary, since dihydro G A_1 is oxidized to ketone with chromic acid, another seems to be tertiary, because of the high stability against the further oxidation with chromic acid. G A_2 methyl ester has, also, two hydroxyl groups and they are proved to be secondary and tertiary, from the results of acetylation and oxidation with chromic acid. From these results and Cross' report[3], it was clarified that G A_1, G A_2 and G acid contain one secondary and tertiary hydroxyl groups. By the selenium dehydrogenation, G A_1 gives 1,7-dimethylfluorene and G A_2 gives a fluorene derivative m.p. 85–90°, which is under investigation at present. From the facts above descrived, it was determined that G A_1, G A_2 and G acid have the same functional groups and skeleton except the numbers of the carbon-carbon double bond (A_1 : 1, A_2 : 0, G acid : 2) and the C-CH_3 (A_1 : 0.5, A_2 : 1.2, G acid : 1.4).

To elucidate the relation between three gibberllins, we attempted the catalytic hydrogenation. From G A_1 methyl ester, a mixture (m.p. 235–7°) of the two dihydro derivatives was obtained, absorbing one mole hydrogen and separated each other only through Al_2O_3 chromatography after acetylation. These acetyl derivatives, which are quite similar but not identical in infra-red spectra, are thought to be stereoisomers each other, which were derived from one asymmetric carbon newly created as the result of hydrogenation. From the G A_2 methyl ester, the unchanged original material was recovered, we expected. From G acid methyl ester, we obtained dihydro G A_1 methyl ester (perhaps a mixture) (ca. 30%) and an acid (ca. 40%), which is under investigation, absorbing two moles of hydrogen. Nevertheless, there should exist theoretically at least four isomers derived from two asymmetric carbons created newly. Cross et al.[2] reported only one tetrahydro G acid methyl ester, m.p. 270–2°. It is unable to decide whether this tetrahydro derivative is the same substance with our dihydro G A_1 methyl ester or not at present. Because, we can not yet obtain each of the deacetylated derivatives from the two stereoisomeric acetyl-dihydro G A_1 methyl ester on account of a small amount of the sample and, also, there is no description of acetyltetrahydro G acid methyl ester by Cross. Moreover, our result that an acid was obtained in relatively good yields might indicate the presence of a lactone ring located at the position to be readily hydrogenolysed such as the alkyl

1) N. Takahashi, H. Kitamura, A. Kawarada, Y. Seta, M. Takai, S. Tamura and Y. Sumiki, This Bulletin **19**, 267, (1955).
2) Cross et al., in press, personal communication.
3) B.E. Cross, *J of Chemical Society*, **1954**, 4670.

oxygen atom of the lactone attached allylically or vinylically with respect to the double bond.

In conclusion, it seems very reasonable to consider that G acid would have one more carbon-carbon double bond in some position of the structure of G A_1, leaving yet the possibility that two dihydro G A_1, G A_2 itself and Cross' tetrahydro G acid are isomers with one another, although dihydro G A_1 methylester was not identical with G A_2 methyl ester in respects of their melting points and infra-red spectra.

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Chemical Structure of Gibberellins. VIII.

(Biochemistry of Bakanae Fungus. Part 38)

Sirs:

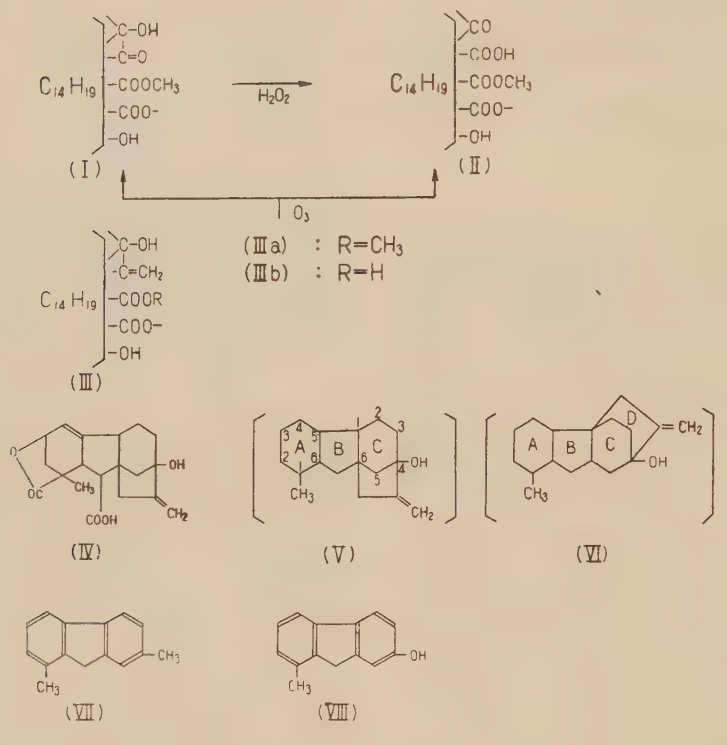
As had been described in previous paper, gibberellin A_1 has one carboxyl, two hydroxyls and one lactone as the functional groups and absorbs 1 mole of hydrogen on catalytic hydrogenation with PtO_2, showing the presence of one carbon-carbon double bond. On the ozonolysis of gibberellin A_1 methyl ester to determine the position of the double bond, we obtained formaldehyde (ca. 45% of theoretical amount), formic acid (33%), a neutral compound, $C_{19}H_{24}O_7$, m.p. 135° (resolidifies and remelts at 170°) (I) and an acid $C_{19}H_{24}O_8$ m.p. 98° (II) (methyl ester m.p. 169°). (I) shows infrared absorption at 1765 (lactone), 1750 (5-membered ring ketone), 1735 (ester carbonyl) cm^{-1} and oxidised with H_2O_2 to (II). It shows negative reaction towards Sciff's reagent but reduces ammoniacal silver nitrate solution or Fehling's solution, suggesting to be α- or β-ketol. Further, (I) is not affected by HIO_4, but its reduction product with $NaBH_4$, m.p. 95–100°, readily consumes HIO_4, producing aldehydic substance. From these facts, (I) is considered to be α-ketol. The nature of the functional groups of (II) are proved to be one carbomethoxyl (infrared absorption at 1732 cm^{-1}), one carboxyl (1703 cm^{-1}), one hydroxyl (mono acetyl derivative of its methyl ester, m.p. 195°) one lactone (1758 cm^{-1}) and one 6-membered ring ketone (1713 cm^{-1} in its methyl ester) from chemical and infrared spectroscopic investigations.

From these results above described, it is assumed that (I) and (II) shall have the partial structures shown in Figures, and, consequently, gibberellin A_1 has the partial structure (IIIb) in the neighbourhood of its ethylenic double bond.

According to the private communication from Dr. Grove (September 7th, 1956), which was a copy to "Chemistry and Industry", he proposed Fig. (IV) as the chemical structure of gibberellic acid (our A_3).

The presence of such a D-ring in gibberellin A_1 as shown in Fig. (V) and (VI) is supported by the following experiments. Gibberellin A_1 gives 1,7-dimethyl fluorene (VII) on Se-dehydrogenation, but from (II), unlike the case of A_1, a hydroxyl compound $C_{14}H_{12}O$, m.p. 159–61°, is obtained which was identified as 1-methyl-7-hydroxy fluorene (VIII) by comparison with synthetic specimen, which was kindly sent us by Dr. J. F. Grove of Imperial Chemical Industries Ltd. England. This fact indicates that one hydroxyl group in gibberellin A_1 must be at 4-position in ring C, and that, at this position, ring D must attach to the ring C. Further, for the aromatization of ring C to give such fluorene derivatives as above described, it will be necessary that another end of ring D must be at 1-or 6-position of ring C. In the former case, newly formed ketone in (I) might be 6-membered ring one, and in the latter case it might be 5-membered ring one. From infrared absorption at 1750 cm^{-1} in (I), this ketone is considered to be 5-membered ring, thus, the superiority of the latter formula is decided. Moreover, the nature of the side chain, locating at 1-position of A-ring is supposed to be either CH_3 or CH_2OH, from the difference of carbon and oxygen. As gibberellin A_1 has one secondary and one tertiary hydroxyl groups, and, as (II) shows (C)-CH_3 number of 0.36, the side chain locating at 1-position is considered to be -CH_3.

This partial structure also interpretes well the fact, that gibberellin A_1 changes to gib-



berellin C by H^+ or Br^- catalysed Wagner-Meerwein rearrangement, on the mechanism of which will be described in the next reports.

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Chemical Structure of Gibberellins. IX.

(Biochemistry of Bakanae Fungus. Part 39)

Sirs:

In the previous papers, three isomers of gibberellin A_1[1]—Gibberellin C[2], isogibberellin A_1[3], and pseudogibberellin A_1[1]—, were reported. In this communication, the relation among these isomers and their partial structures are presented.

When gibberellin A_1 is treated with mineral acid, gibberellin C is isolated which has one hydroxyl, one carbonyl, one lactone, and one carboxyl groups but no ethylenic double bond. In the course of this reaction, it has been found that one hydroxyl group, perhaps tertiary, and one double bond in gibberellin A_1 are converted to a carbonyl group in gibberellin C.

By the alkaline treatment of gibberellin C, isogibberellin A_1 is obtained which has the same functional groups as gibberellin C and is thought to be an epimer of gibberellin C.

When gibberellin A_1 methyl ester is treated with aqueous alkaline solution, pseudogibberellin A_1 is obtained which is thought to be an epimer of gibberellin A_1, having the same functional groups.

By treating pseudogibberellin A_1 with acid, isogibberellin A_1 is obtained. From the result above described, it seems to us that the relationship between these four isomers can be clarified and is summarized in Fig. 1.

When the gibberellin A_1 is treated with bromine-dioxane complex, a monobromogibberelline A_1 is obtained which has one hydroxyl, one carbonyl, one lactone, one carboxyl groups. This monobromogibberellin A_1 is readily converted into gibberellin C by the treatment of zinc-acetic acid, or catalytic reduction in alkaline media using $Pd\text{-}CaCO_3$ as its catalyser, but not dehydrobrominated in boiling collidine recovering the original substance. As in the case of preparation of gibberellin C from gibberellin A_1, this fact shows that one of two hydroxyl groups and a double bond in gibberellin A_1 are converted to a carbonyl group.

gibberellin A_1	$\xrightarrow{OH^-}$	pseudogibberellin A_1
$C_{19}H_{24}O_6$ d.p. 232–5°		$C_{19}H_{24}O_6$ m.p. 225–7°
2 –OH		2 –OH
1 –COOH		1 –COOH
1 –COO–		1 –COO–
1 double bond		1 double bond
$\downarrow H^+$		$\downarrow H^+$
gibberellin C	$\xrightarrow{OH^-}$	isogibberellin A_1
$C_{19}H_{24}O_6 \cdot H_2O$ d.p. 252–4°		$C_{19}H_{24}O_6$ d.p. 260–2°
1 –OH		1 –OH
1 >CO		1 >CO
1 –COOH		1 –COOH
1 –COO–		1 –COO–
no double bond		no double bond

FIG. 1.

The bahaviour of gibberellin A_1 to acid (H^+) and bromine (Br^+) leads us to a conclusion that in the course of these reaction the formation and rearrangement of intermediate carbonium cation are involved.

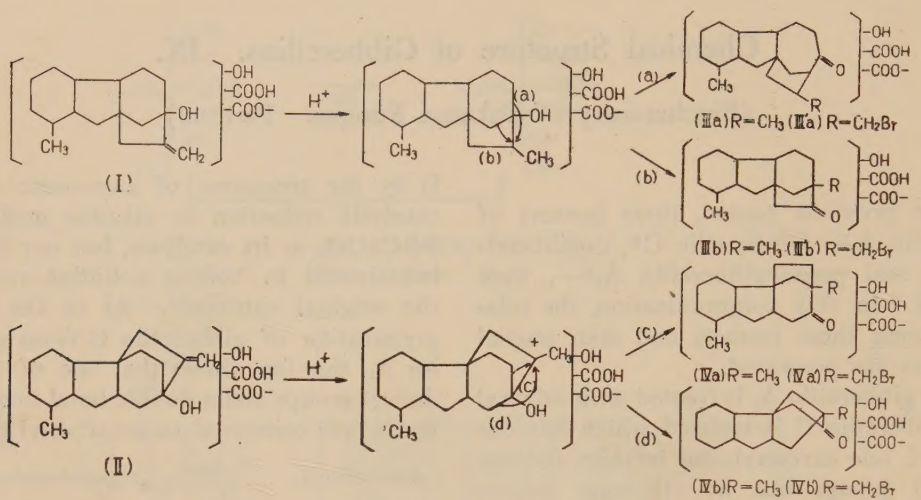
If we accept (I) or (II) as the partial structure of gibberellin A_1 and consider the mechanism of rearrangement, the four partial structures (IIIa), (IIIb), (IVa) and (IVb) might be proposed to gibberellin C and the four (IIIa′), (IIIb′), (IVa′), (IVb′) to monobromogibberellin A_1.

But on the dehydrogenation by selenium, both gibberellin A_1 and C give 1,7-dimethylfluorene and no other hydrocarbon is isolated in crystalline forms. This result can be in-

1) N. Takahashi, A. Kawarada, H. Kitamura, Y. Seta, M. Takai, S. Tamura and Y. Sumiki, This Bulletin, **19**, 267 (1955).

2) A. Kawarada, H. Kitamura, Y. Seta, N. Takahashi, M. Takai, S. Tamura and Y. Sumiki, *ibid.*, **19**, 278 (1955).

3) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igarashi, and K. Tamari, *J. of Agri. Chem. Soc. of Japan*, **17**, 894 (1940).



terpreted by the structure (I) as gibberellin A_1 and (IIIb) as gibberellin C successfully, other formulae being discarded. The fact that monobromogibberellin A_1 is not dehydrobrominated in boiling collidine comfirms the hypothetical formula (IIIb′) as monobromogibberellin A_1.

Cross et al.[4] proposed the similar partial structure for gibberic acid, which is acid degradative product of gibberellic acid, from the view point of the same mechanism of rearrangement.

On the bromination of gibberellin C in acetic acid at 60°, one mole of bromine is absorbed and no more bromine is absorbed and a monobromogibberellin C is obtained as its resulting compound. If gibberellin C has such a ring ketone system as shown in structure (IIIb), the substitution of second bromine atom might be hindered sterically.

4) Cross et al, in press. personal communication.

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